IODOTHYRONINE DEIODINASES: STRUCTURE-FUNCTION ANALYSIS AND THEIR ROLE IN THE REGULATION OF THYROID HORMONE LEVELS

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JODOTHYRONINE DEJODASES: STRUCTURE-FUNCTIE ANALYSE EN HUN ROL IN DE REGULATIE VAN SCHILDKLIERHORMOON SPIEGELS

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Contents

List of abbreviations

ACE	angiotensin converting enzyme
ANF	atrial natriuretic factor
BrAc	N-bromoacetyl
BSA	bovine serum albumin
BW	body weight
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxynucleic acid
CDS	coding sequence
CHF	congestive heart failure
CHT	congenital hypothyroidism
CNS	central nervous system
cpm	counts per minute
D1	type I iodothyronine deiodinase
D2	type II iodothyronine deiodinase
D3	type III iodothyronine deiodinase
Da	dalton
DEHAL1	iodotyrosine deiodinase
DEPC	diethylpyrocarbonate
DIT	3,5-diiodotyrosine
DNA	deoxynucleic acid
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
EST	expressed sequence tag
FAT	fatty acid translocase
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GPX	glutathione peroxidase
GTG	goldthioglucose
HPLC	high performance liquid chromatography
HYP	compensatory cardiac hypertrophy
IAc	Iodoacetate
IC_{50}	concentration causing 50% inhibition
IOP	iopanoic acid
IRD	inner ring deiodination
K_m	Michaelis Menten constant
LV	left ventricle
MCT	monocrotaline
MCT8	monocarboxylate transporter 8
MHCα/β	myosine heavy chain alfa/beta
MIT	3-monoiodotyrosine
mRNA	messenger ribonucleic acid
NIS	Na/I symporter
NTI	nonthyroidal illness (sick euthyroid syndrome)
ORD	outer ring deiodination
PCR	polymerase chain reaction
PLB	phospholamban
PTH	parathyroid hormone
PTU	propylthiouracil

RIA	radioimmunoassay
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
rT3	reverse T3 (3,3’,5’-triiodothyronine)
RV	right ventricle
RXR	retinoid X receptor
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SeC	selenocysteine
SECIS	selenocysteine insertion sequence
SEM	standard error of the mean
SERCA	sarcoplasmic reticulum calcium ATPase
SNP	single nucleotide polymorphism
T0	thyronine
T1	monoiodothyronine
T2	(3,3’-)diiodothyronine
T3	(3,3’,5-)triiodothyronine
T4	thyroxine (3,3’,5,5’-tetraiodothyronine)
T4S, rT3S,T3S, T2S	sulfated thyroid hormone metabolites
TBG	thyroxine-binding globulin
TBPA	thyroxine-binding prealbumin (transthyretin)
TG	thyroglobulin
THOX1 and 2	thyroid oxidase 1 and 2
TPA	12-O-tetradecanoylphorbol-13-acetate
TPO	thyroperoxidase
TR	thyroid hormone receptor
TRE	thyroid responsive element
TRH	thyrotropin releasing hormone
TSH	thyroid stimulating hormone (thyrotropin)
UTR	untranslated region

CHAPTER 1

GENERAL INTRODUCTION

THE THYROID GLAND

The human thyroid gland initially forms as a midline outpouching of the anterior pharyngeal floor, visible at 16-17 days gestation. Lateral contributions, i.e., the ultimobranchial bodies, develop as caudal projections from the fourth to the fifth pharyngeal pouches. The thyroid gland migrates caudally, remaining attached to the pharyngeal floor by the thyroglossal duct, reaches its final position by 7 weeks gestation, and its definitive bilateral shape by the association between the medial and lateral thyroid anlage by 8-9 weeks gestation. During its caudal displacement, it leaves the thyroglossal duct, which usually becomes fragmented and resorbed, but may persist to form thyroglossal cysts. If migration is incomplete, ectopic thyroid tissue may lie at the base of the tongue. The normal adult thyroid gland weighs about 20 g, contains 2 lobes joined by an isthmus and lies in front of the thyroid cartilage (1). Histologically, fibrous septa divide the gland into pseudolobules, which, in turn, are composed of vesicles, called follicles or acini, surrounded by a capillary network. Normally, the follicle walls are composed of cubical epithelium. The lumen is filled with a proteinaceous colloid, which contains a protein peculiar to the thyroid, thyroglobulin, within the peptide sequence of which thyroxine (T4) and 3,3',5'-triiodothyronine (T3) are synthesized and stored. Thyroid follicles undergo three stages of development, the precolloid stage (7-13 weeks), the colloid-onset stage (13-14 weeks), and the follicular stage (from week 14 onwards). Thyroglobulin is detected as early as in the 5^{th} gestational week. Active trapping of iodide by the thyroid is detectable by the 12^{th} week, during the precolloid stage, and the first indication of T4 production occurs 2 weeks later, during the final stage of follicular lumen formation. Fetal serum T4 concentrations remain at very low levels until midgestation; at 18-20 weeks both the fetal thyroid gland iodine uptake and serum T4 concentrations begin to increase (2-5). The thyroid contains a smaller second population of cells, the parafollicular or C-cells, which lie between the follicles. They secrete the calcium-lowering hormone calcitonin and together with the parathyroid hormone (PTH) secreting parathyroid glands, they play a role in the regulation of Ca^{2+}-homeostasis (1, 6, 7).

THE HYPOTHALAMIC-PITUITARY-THYROID AXIS

In the hypothalamus, thyrotropin-releasing hormone (TRH) is detected by 8-9 weeks gestation, and its content increases progressively thereafter. The hypothalamic-hypophyseal vascular portal system is histological detectable by 9 weeks, and thyroid stimulating hormone (TSH) is present in the pituitary by 10-12 weeks. Prior to this time, the fetal thyroid is not able to organify iodine (6). The growth and function of the thyroid gland is controlled by a at least four mechanisms: a) the classic hypothalamic-pituitary-thyroid axis, in which TRH, synthesized by neurons in the paraventricular nuclei of the hypothalamus and stored in the median eminence, is then transported via the pituitary portal venous system down the pituitary stalk to the anterior pituitary gland, where it stimulates the synthesis and release of anterior pituitary TSH, which in turn stimulates growth and hormone secretion by the thyroid gland. This results in a rise in serum thyroid hormone levels, with thyroid hormones exerting a negative feedback action at hypothalamic and pituitary level. This negative feedback action can be performed directly by T3, or indirectly by T4, that has to be converted in the pituitary and hypothalamus to T3 to exert its action (Fig. 1); b) TSH synthesis and release can be inhibited by other hypothalamic hormones and drugs, such as somatostatin, dopamine, dopamine agonists such as bromocriptine, and glucocorticoids; c) autoregulation of hormone synthesis by the thyroid gland itself in relationship to its iodine supply; d) stimulation or inhibition of thyroid function by TSH receptor autoantibodies (7). However, this last mechanism is only observed in disease states, like M. Graves.

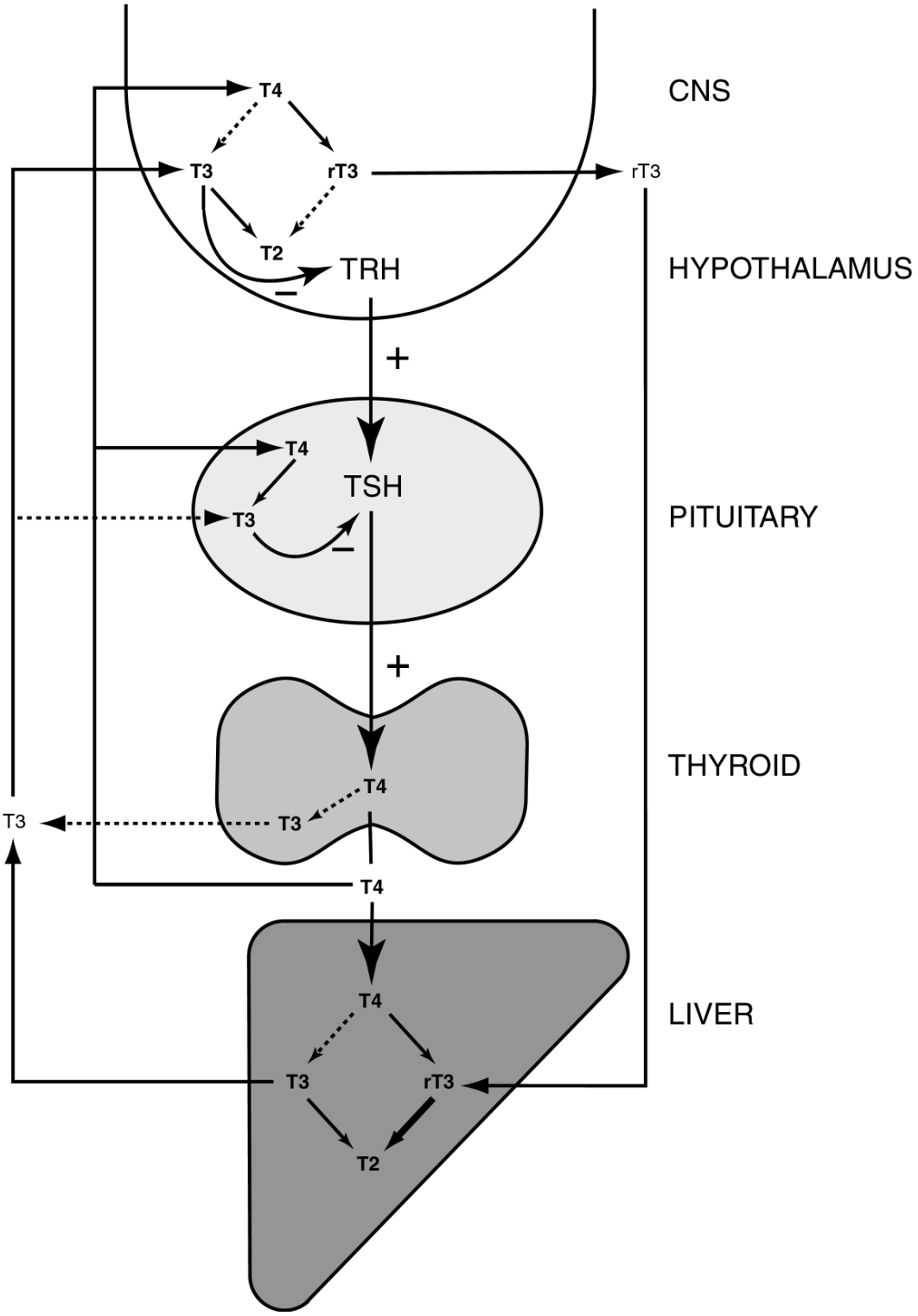

Fig.1. The hypothalamic-pituitary-thyroid axis

THYROID HORMONES IN PATHOPHYSIOLOGY

Thyroid hormones are important for energy metabolism, the metabolism of nutrients and inorganic ion fluxes, thermogenesis, cardiac performance, and for stimulation of growth and development of various tissues, including the central nervous system, at critical periods (8). Whereas in the adult, thyroid hormone deficiency or excess may lead to an extensive array of clinical manifestations, including neurological and psychiatric symptoms (9, 10), which are usually reversible with proper treatment, prolonged deficiency of thyroid hormones during development usually leads to irreversible damage, depending on the specific timing of onset and duration of thyroid hormone deficiency. The most obvious abnormalities associated with thyroid hormone deficiency, i.e., goiter (thyroid gland enlargement) and cretinism (a form of mental deficiency, together with defects in skeletal growth) were recorded as early as 2000 B.C. (11). The physiological effects of thyroid hormones are largely mediated by varying patterns of genomic activation and protein synthesis. Thyroid hormones potentiate the actions of catecholamines, and their effect on somatic and skeletal growth are in part mediated by stimulation of the synthesis and action of growth hormone and insulin-like growth factor I (6-8).

Thyrotoxicosis, i.e., conditions in which tissues are exposed to high levels of thyroid hormone, is a clinical entity where the effects of an excess of thyroid hormone can be studied. In most cases, thyrotoxicosis is due to hyperactivity of the thyroid gland, or hyperthyroidism. Graves' disease is the most common cause of hyperthyroidism (12, 13). It is an autoimmune disorder characterized by hyperthyroidism, diffuse goiter, often ophtalmopathy and, rarely, dermopathy. The hyperthyroidism and goiter of Graves' disease are caused by stimulation of the thyroid by TSH receptor antibodies and production of these antibodies occurs within the thyroid gland itself. In common with other autoimmune diseases, genetic, environmental and endogenous factors are required in an appropriate combination to initiate thyroid autoimmunity. The rate of concordance for Graves' disease is about 20 percent among monozygotic twins, and the rate is much lower among dizygotic twins, indicating that genes make only a moderate contribution to susceptibility (14). No single gene is known to cause the disease or to be necessary for its development. There is a well-established association with certain HLA alleles that varies among racial groups (15, 16). Furthermore, Graves' disease is associated with polymorphisms of the cytotoxic T-lymphocyte antigen 4 (CTL4) gene in several racial groups (14, 17). There is no clear genetic susceptibility to the development of ophtalmopathy (18). Environmental factors known to predispose to autoimmune thyroid

disease include smoking, stress and iodine intake (including amiodarone). The thyroid itself finally appears to play a major role in disease progression, interacting with the immune system through expression of a number of immunologically active molecules including HLA class I and II, adhesion molecules, cytokines, CD40 and complement regulatory proteins (19). Female sex (sex steroids), the postpartum period, lithium therapy, and some rare factors (such as interferon-α therapy, antiretroviral therapy for HIV infection, Campath 1-H monoclonal antibody for multiple sclerosis) are also considered to be predisposing factors for Graves' disease. It has been suggested that a genetic clonal lack of suppressor T cells may be responsible for the onset in production of TSH receptor antibody (20).

When thyrotoxicosis, goitre and ocular signs and symptoms coexist, the diagnosis of Graves' disease appears self-evident. However, 50% of patients with Graves' disease may not have clinically detectable ophtalmopathy at presentation, making the diagnosis less obvious (21). The clinical features are shown in table 1. Many manifestations of hyperthyroidism, including palpitations, a tremor, and anxiety, are due to increased adrenergic tone and may be confused with an anxiety disorder (22). Elderly patients commonly present in an atypical fashion with only weight loss and anorexia or isolated atrial fibrillation. They also tend to have their symptoms for longer periods, have smaller multinodular goiters and do not have ocular signs or symptoms (23, 24). In younger individuals, common manifestations include palpitations and tremor, nervousness, easy fatigability, hyperkinesia, and diarrhea. These symptoms are indicative for the important chronotropic and inotropc effects of thyroid hormone on the heart, its effects on the sympathetic nervous system, and its gastrointestinal action. Other symptoms are excessive sweating, intolerance to heat, and preference to cold. There is often weight loss without loss of appetite. These phenomena can be attributed to the fact, that thyroid hormone is a thermogenic hormone and thermogenesis is energy dissipation as heat. Furthermore, the thermogenic effect of thyroid hormones is closely linked to increased appetite and lipogenesis to ensure fuel availability and to avoid wasting (25). Since hyperthyroidism increases hepatic gluconeogenesis and glycogenolysis as well as intestinal glucose absorption it will exacerbate underlying diabetes mellitus. In lipid metabolism it stimulates lipolysis and there is a cholesterol lowering effect, whereas in hypothyroidism hypercholesterolemia can be seen. Neuromuscular effects are described as well, as there is muscle weakness or myopathy, i.e. an increased protein turnover and loss of muscle tissue, and there also is an increase in speed of muscle contraction and relaxation. In children, rapid growth with accelerated bone maturation occurs.

Table 1. Clinical features of Graves' disease (see references (26, 27))

SYMPTOMS	SIGNS
HYPERTHYROIDISM	
dyspnea on exertion	struma
fatigue and weakness	warm, moist hands
palpitations	regular pulse >90
heat intolerance	tremor of fingers
excessive sweating	arrhythmias/tachycardia
nervousness	onycholysis
tremulousness/tremor	palmar erythema
weight loss	thinning of the hair
↑ appetite	hyperactive reflexes
oligo-/amenorrhea	proximal myopathy
erectile dysfunction/gynecomastia	hyperkinesis
diarrhea	dermatopathy (rare)
high urinary frequency/nocturia/thirst	stare/eye lid retraction/bright, shiny eyes
anxiety/emotional lability/insomnia	
restlessness/inability to concentrate	
OPHTALMOPATHY	
irritation/dryness/tearing	periorbital edema
visual blurring	conjunctival erythema
diplopia	chemosis (conjunctival edema)
pain on eye movement	proptosis
visual loss	ophtalmoplegia
	loss of color vision (optic neuropathy)
	papilledema (optic neuropathy)

The diagnosis of hyperthyroidism is generally straightforward, since serum thyrotropin (TSH) is low or, in most cases, suppressed. Together with elevated free thyroxine levels (FT4) hyperthyroidism is confirmed. However, 10% of patients will have an increased total or free T3 level in the face of a normal FT4 and suppressed TSH level, a condition referred to as T3 toxicosis (28). Whereas TSH-R autoantobodies are usually present, antibodies to thyroglobulin and thyroid peroxidase may be present as well but are not diagnostic (27).

Three treatment modalities are available, i.e. antithyroid drug therapy, surgery, and radioactive iodine uptake (RAIU) therapy. All effectively restore euthyroidism but have potentially serious side effects. Since increased β-adrenergic activity is responsible for the palpitations, tachycardia, the tremor, anxiety and heat intolerance, β-blockers are effective adjuvant therapeutics. Although some impair T4-to-T3 conversion, it is unlikely that this effect is of clinical significance. RAIU therapy has become the preferred treatment for adults

with Graves' disease. It is administered orally as iodine 131 in solution or capsule. Radioactive iodine is rapidly incorporated into the thyroid and via its β-emissions produces tissue necrosis resulting in euthyroidism usually within 6-18 weeks (27). There is no association of radioiodine with birth defects, infertility or overall cancer incidence (29). Therefore, it is easy to administer, safe, effective, and more affordable than long-term treatment with antithyroid drugs. However, hypothyroidism is an inevitable consequence of RAIU therapy. This therapy is contraindicated in pregnant women, because it damages the fetal thyroid gland, resulting in fetal hypothyroidism.

In pregnancy antithyroid drug therapy is indicated. Although the antithyroid drugs propylthiouracil (PTU) and methimazole (MMI) have similar efficacy, PTU has historically been the drug of choice for treating pregnant and breast-feeding women because of its limited transfer into the placenta and breast milk. However, there is ample evidence that methimazole should be the antithyroid drug of choice in the non-pregnant patient, since methimazole is more effective at equivalent doses, and decreases thyroid hormone levels more rapidly thereby attaining euthyroidism sooner (30, 31). Furthermore, it has a longer half-life and thus can be used as a single daily agent, and this is more likely to be associated with patient compliance. Finally, and most importantly, methimazole would appear to have a more favorable safety profile, since agranulocytocis, hepatitis and vasculitis occur more commonly with propylthiouracil (30, 32). Although several recent reviews state that the remission rate following antithyroid drugs is in the 30-40% range (18, 33), this opinion has been challenged by others stating that there is only a modest hope of achieving a permanent remission (27). This, together with the side effects of antithyroid drugs, the high relapse rate and the fact that a patient should receive a course of antithyroid drugs for 1-2 years before determining whether an immunological remission has occurred make them a less favorable therapeutic option (27).

Finally, bilateral subtotal thyroidectomy, which was once the only treatment available, is now performed only in special circumstances. It should be considered in patients with large goitres, when patients are intolerant of antithyroid drugs and/or choose to refuse radioiodine therapy, during the second trimester of pregnancy after failure of antithyroid drugs, or when there is concern about worsening of ophtalmopathy following radioiodine. In addition to the normal risks associated with surgery, recurrent laryngeal nerve paresis, hypoparathyroidism, and hypothyroidism can occur following this procedure (13). In order to reduce the risk of thyroid storm, patients should be rendered euthyroid before surgery (27).

THYROID HORMONE SYNTHESIS

The unique feature of the thyroid hormones is their content of iodine. The thyroid gland contains about 90% of the body's total iodine, mainly in organic form (1). The thyroid secretes T4 and T3 in a proportion determined by the T4/T3 ratio in Tg (15:1 in humans) as modified by the thyroidal conversion of T4 to T3 (34). Thus, the prohormone T4 is the major secreted iodothyronine in iodine-sufficient subjects, with the ratio of secreted T4 to T3 being about 11:1 (35). The synthesis of T4 and T3 may be characterized as follows (36) (Fig. 2):

A) Active transport of iodide into the cells by a process that is competitively inhibited by other anions of similar size, such as perchlorate (ClO_4^-) and pertechnate (TcO_4^-). The site of this active transport of iodide in thyroid epithelial cells is most likely the basal membrane (37). This active transport into the thyroid cell (trapping of iodine) is markedly stimulated by TSH, TSH receptor stimulating antibody as found in M. Graves and other stimulators of the cAMP cascade, whereas mitogens like epidermal growth factor and 12-O-tetradecanoylphorbol 13-acetate (TPA) inhibit the iodide trapping mechanism. Both latter substances dedifferentiate thyroid follicular cells (38). The transport of iodide is mediated by the Na/I symporter (NIS). Whereas NIS regulates I^- transport at the basolateral membrane, I^- transport at the apical membrane, i.e. from the cell to the colloid space, is performed by a protein named pendrin (39, 40). ClO_4^- has been used clinically in combination with thionamides to treat severe forms of hyperthyroidism and in combination with ^{123}I to demonstrate organification defects in the thyroid gland. Two to 3 hours after administration of ^{123}I, ClO_4^- is administered orally, blocking further active transport of I^- into the thyroid cell. Whereas in the normal subject no significant decrease in radioactivity is detectable over the thyroid gland, a significant decrease in radioactivity can be observed in case of an organification defect due to discharge of thyroidal I^- (7, 36). $^{99m}TcO_4^-$ is used as a radiolabel to visualize the thyroid and also to measure the activity of the "iodide pump". Following uptake, $^{99m}TcO_4^-$ is not covalently bound to proteins in the thyroid and therefore solely measures the transport process.

B) Iodination of tyrosyl residues in thyroglobulin (TG) is the next step involved. The essential steps in this process require, apart from iodide and TG, a peroxidase, i.e. thyroperoxidase (TPO) and supply of H_2O_2. TPO needs H_2O_2 for oxidation. Human thyroglobulin (TG) is a homodimeric glycoprotein (10% carbohydrate) with a molecular weight of 670 kDa. It contains about 140 tyrosyl residues of which only 40 are available for iodination and only a few are involved in hormone synthesis. After its synthesis in the

follicular cell, TG is stored in the follicular lumen and oxidation of iodide to iodine and incorporation into mono- and di- iodotyrosine (MIT and DIT) residues on TG takes place at the border of the apical membrane of the follicular cell (Fig. 2).

C) Coupling of the iodinated phenol of one iodotyrosine residue to the phenol hydroxyl group of another to form T4 or T3. When MIT joins with DIT, T3 is formed, and when DIT joins with DIT, T4 is formed. These reactions are catalyzed by TPO that is also involved in tyrosyl iodination. Thiocarbamide drugs, particularly PTU, methimazole and carbimazole, are potent inhibitors of TPO and therefore will block thyroid hormone synthesis. These drugs are clinically useful in the management of hyperthyroidism.

D) Endocytosis of TG from the follicular lumen, proteolysis by lysosomal hydrolases, with release of free iodotyrosines and iodothyronines, and secretion of iodothyronines into the blood.

E) Deiodination of iodotyrosines within the thyroid cell with conservation and reuse of the liberated iodide ("intrathyroidal iodide cycle"). Iodotyrosine deiodinase, i.e. DEHAL1, recently has been characterized (41).

F) Intrathyroidal outer-ring deiodination (ORD) of T4 to T3. Whether passive diffusion or an active transport mechanism is involved in the release of T3 and T4 out of the thyrocyte into the blood stream remains unclear.

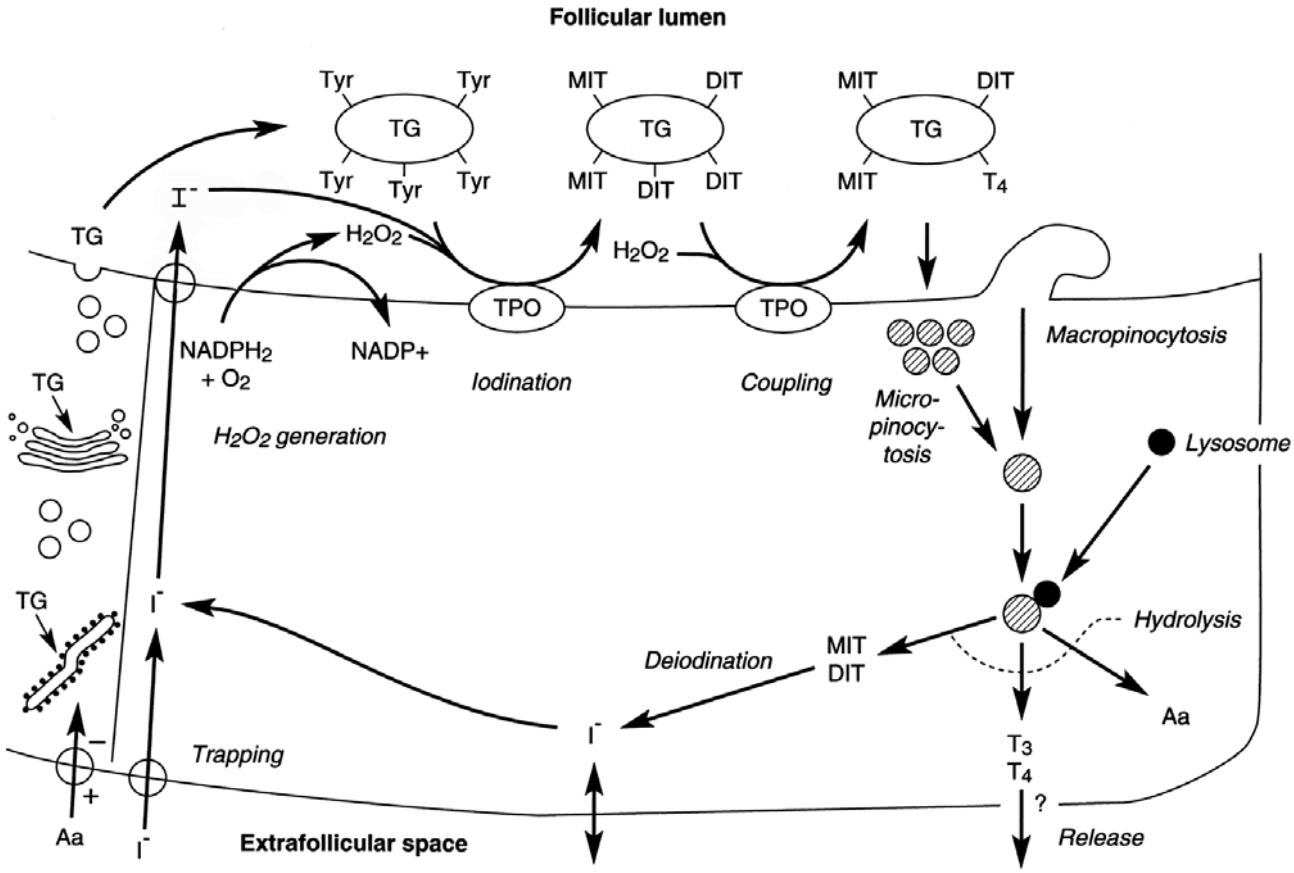

Fig. 2. Thyroid hormone biosynthesis in the follicular cell

Defects in thyroid hormone biosynthesis have been well described and can lead to permanent congenital hypothyroidism (CHT). CHT is the most prevalent endocrine disorder in the newborn and affects 1 in 3,000-4,000 newborns, with the exception of the African-American population in the USA where the incidence is 1 in 10,000 (42). Screening for CHT is a major achievement of pediatrics because early diagnosis and treatment have resulted in normal development in nearly all cases. Since 1981 the screening program in The Netherlands consists of determination of T4 levels 5-7 days after birth, followed by measurement of TSH whenever T4 values deviate. Permanent CHT can be divided in CHT with a normally developed thyroid gland (20%-25%), i.e. defective thyroid hormone biosynthesis, and with a defectively developed thyroid gland (75%-80%). Central hypothyroidism is a rare disease affecting 1 in every 20,000 newborns (43). Overall, these cases represent a small percentage of the population with CHT, and the cause of the vast majority remains unknown (44, 45). Examples of transient CHT, with only a transient need for thyroid hormone suppletion, are iodine deficiency, exposure to excess iodine in the perinatal period, or fetal exposure to either

maternally derived thyroid-blocking antibodies or antithyroid drugs taken by pregnant women with thyroid autoimmune disease. Prematuritas can also be a cause of transient congenital thyroid dysfunction.

The genetic background for the defects resulting in defective thyroid hormone biosynthesis is well accepted. There is an autosomal recessive inheritance of loss-of-function mutations in candidate genes like the NIS gene (46, 47), the TG gene (48, 49), the pendrin gene (50, 51), and the TPO gene (52-54). So far, 21 mutations in ten exons have been described confirming the heterogeneity of TPO defects (55-58). Attention to the importance of a molecular genetic diagnosis in CHT patients with proven defects of TPO has been emphasized since the recently reported thyroid follicular carcinoma in a newborn with a heterozygous mutation in exon 14 of the TPO gene (59), and follicular adenoma in a patient with compound heterozygous mutations in exons 7 and 14 (56). Only recently, it became possible to examine the molecular basis of congenital hypothyroidism due to an iodide transport defect (ITD). To date about 50 cases of ITD, corresponding to 33 families, have been reported worldwide. Seventeen cases from 13 families studied at the molecular level have been shown to have a mutation in NIS. Nine mutations have been identified (60). Although the clinical picture has been well described, the molecular mechanisms underlying the effects of most of the mutations are still not clear.

Besides the fact that NIS provides the basis for the effective diagnostic and therapeutic management of thyroid cancer and its metastases, the discovery of endogenous NIS expression in more than 80% of human breast cancer samples has raised the possibility that radioiodide may be a valuable novel tool also in breast cancer diagnosis and treatment (39).

Pendred's syndrome, an autosomal recessive condition characterized by congenital sensoneurinal hearing loss and goiter, is caused by mutations in the PDS gene. It encodes a chloride-iodide transporter called pendrin expressed in the thyroid, the inner ear and kidney. As mentioned before, pendrin is probably devoted to regulate the flux of iodide from the thyroid cell to the colloid space at the apical membrane. In the inner ear, a pronounced PDS expression has been detected in structures like the membranous labyrinth and the endolymphatic duct and sac and the function of pendrin could be the maintenance of the appropriate ionic composition of the endolymph (40, 61).

Recently, inactivating mutations in the gene for Thyroid Oxidase 2 (THOX2) have been described with biallelic mutations associated with severe and permanent CHT, whereas monoallelic mutations were associated with milder, transient hypothyroidism (62). Together with the Thyroid Oxidase 1 (THOX1) protein the THOX2 protein has recently been identified

as a component of the H_2O_2 generation system of the thyroid (63, 64). As mentioned before, the generation of H_2O_2 is a critical step in the synthesis of thyroid hormones, because it is an essential component in the iodide organification process.

The most common cause of permanent CHT is thyroid dysgenesis. This ranges from athyrosis, without visible thyroid tissue, thyroid ectopy most frequently located in a sublingual position and hypoplasia with remnants of thyroid tissue in the normal position, to hemithyroidea with the presence of a single lobe only (42). Different factors control the formation of the thyroid gland during the phases of induction, migration, bifurcation, proliferation and differentiation. Using in situ hybridization in mouse embryos three transcription factors, involved in the thyroid organogenesis, have been identified: Pax-8, TTF-2 and NKX2.1. However, none of these factors are exclusively expressed in the thyroid and therefore there is an association with developmental defects in other organs. According to the studies of targeted gene disruption in mice (65) and mutations of transcription factors in patients with CHT (66), athyrosis, hypoplasia and ectopy can be regarded as different degrees of severity of the same molecular defects (65).

Whereas there is a dominant inheritance of the mutations encoding for transcription factors, it was shown in mice that the autosomal recessive inheritance of loss-of-function mutations of the thyroid stimulating hormone (TSH) receptor (P556L) led to thyroid hypoplasia (42). Screening of the TSH receptor gene in CHT patients yielded several loss-of-function mutations resulting in a spectrum of severity ranging from a relatively mild resistance to TSH (67) to apparent athyrosis on a thyroid scan (68, 69).

Although Pax-8 is also expressed in the kidney and central nervous system, homo- or heterozygous knockout animals did not show any abnormalities of these organs. Furthermore, screening in CHT patients for Pax-8 mutations has led to the identification of several patients with heterozygous mutations that have been inherited in a dominant fashion (66, 70, 71). A hypoplastic thyroid with a cystic appearance is seen on ultrasound. Ectopicity was described in one patient. The severity of the hypothyroidism was mild to moderate.

In TTF-2 knockout mice both athyrosis and thyroid ectopy were identified, whereas these mice also have a cleft palate which makes feeding impossible and early neonatal death unavoidable (65). Screening for the TTF-2 gene in a CHT patient population did not reveal any mutation (72). However, a mutation in the aforementioned gene could be identified in the so called "Bamforth syndrome" (73). This is a particular syndrome of CHT, developmental delay, cleft palate, choanal atresia and spiky hair. Homozygosity of a loss-of function mutation in two siblings was demonstrated, whereas the heterozygous parents were unaffected

(74). Therefore, TTF-2 mutations seem to be a very rare cause of CHT in humans, resulting in a specific syndrome with other organ manifestations.

The NKX2.1 gene encodes for a transcription factor, which has been shown to be expressed during embryonic development in the thyroid, hypothalamus, basal ganglia, pituitary and lung in mice. Targeted disruption of this gene leads to a complex phenotype of newborn mice (75), who die shortly after birth due to respiratory distress from defective lung development with insufficient surfactant production. Whereas homozygous newborn mice were athyroid, heterozygous mice exhibited no abnormalities of thyroid development. Early death prevented a more thorough study of hypothalamic-pituitary function whereas neurological testing also could not be performed. Screening of CHT patients for mutations in the NKX2.1 gene did not show any abnormalities (44). However, two studies reported on patients with presenting symptoms of mild CHT, pulmonary problems, unexplained ataxia and muscular hypotonia, with an underlying deletion of chromosome 14, including the NKX2.1 locus (76, 77). Thereafter, further 6 patients with variable degrees of CHT who suffered from choreoathetosis, muscular hypotonia, and pulmonary problems were described (78, 79). Heterozygous loss of function mutations in 5 of these patients were discovered, e.g. one complete gene deletion, one missense mutation, and three nonsense mutations. Despite early treatment at an adequate dosage the outcome of the development of these patients was unfavorable. The association of symptoms in the patients with NKX2.1 mutations points to an important role of human NKX2.1 in the development and function of thyroid, basal ganglia, and lung, as already described for rodents, and the unfavorable outcome in these patients therefore can be explained by these mutations rather than by hypothyroidism. Thus, a new syndrome of CHT, pulmonary complications and choreoathetosis can be attributed to mutations of the NKX2.1 gene (42).

THYROID HORMONE TRANSPORT

Thyroid hormones circulate in the serum bound to carrier proteins. There are 3 major thyroid hormone transport proteins, i.e. T4-binding globulin (TBG), transthyretin (TTR), formerly known as thyroxine-binding prealbumin (TBPA), and albumin. In humans, TBG, TTR and albumin carry about 75%, 15% and 10% of plasma T4 and T3, respectively (36, 80-82) Since normal adult rats lack TBG, TTR is the main plasma transport protein in rat serum (82).

Although in normal human serum free T4 (FT4) and free T3 (FT3) only comprise 0.02% and 0.2% of total T4 and T3, it is this free fraction that is responsible for hormonal action. Since thyroid hormones are fat soluble it was originally believed that thyroid hormone enter target cells by passive diffusion. Now it is clear that carrier mediated processes are necessary for cellular uptake. Uptake of T4 and T3 is energy, temperature, and often sodium dependent, and represents the translocation of thyroid hormone over the plasma membrane (83). The thyroid hormone transporters may differ in the different organs. In the liver there are different T3 and T4 transporters (84-86), while in the pituitary there is a common transporter for T3 and T4 (87-89).

Whereas initially only T3 and not T4 transport could be identified in the heart (90), it was shown recently, using the Xenopus laevis (X. laevis) expression system (91, 92), that rat Fatty Acid Translocase (rFAT) transports T3, T2, rT3, T4 and T3S (93). Since the heart depends on uptake of fatty acids for supplying around 70% of the energy demand, FAT could be an important mechanism in cardiac transport of iodothyronines. The contribution of fatty acids to cardiac energy production may change depending on the subject's activity or under pathological conditions (93).

Several organic anion transporters and L-type amino acid transporters have been shown to facilitate the cellular uptake of thyroid hormone (94-100). Recently, monocarboxylate transporter 8 (MCT8), a homologue of a T-type amino acid transporter, was identified as a very active and specific thyroid hormone transporter with high expression in liver, kidney, brain and heart (101). Mutations in MCT8 have been identified in young male patients with a novel syndrome of severe psychomotor retardation and strongly elevated serum T3 concentrations. The MCT8 gene is located on the X-chromosome, and hemizygous mutation of this transporter is thought to result in a defect in thyroid hormone uptake into the brain, which is detrimental for neurological development (102, 103)

THYROID HORMONE METABOLISM

Thyroxine undergoes multiple metabolic reactions. The most important of these is deiodination, especially because of its role in the regulation of thyroid hormone bioactivity. In addition to deiodination, iodothyronines are metabolized by conjugation of the phenol hydroxyl group with sulfate or glucuronic acid (104) (Fig. 3), and, to a minor extent, by ether bond cleavage (105) and oxidative deamination of the alanine side chain (106). The latter converts T4 to Tetrac (TA4) and T3 to Triac (TA3). The general purpose of sulfation and

glucuronidation is to increase water solubility of the substrates in order to facilitate their urinary and biliary clearance. However, only a small amount of iodothyronine sulfates normally appears in urine, bile or serum, because these compounds are rapidly deiodinated by the type I iodothyronine deiodinase (D1). In particular, the tyrosyl ring deiodination (inner ring deiodination, IRD) of T4S and T3S is strongly enhanced, suggesting that sulfate conjugation is a primary step leading to irreversible inactivation of thyroid hormone (104, 107). In contrast to the sulfates, iodothyronine glucuronides are rapidly excreted in the bile. However, this is not an irreversible pathway of hormone disposal, since after hydrolysis of the glucuronides by bacterial β-glucuronidases in the intestine at least part of the liberated iodothyronines are reabsorbed, constituting an enterohepatic cycle (104).

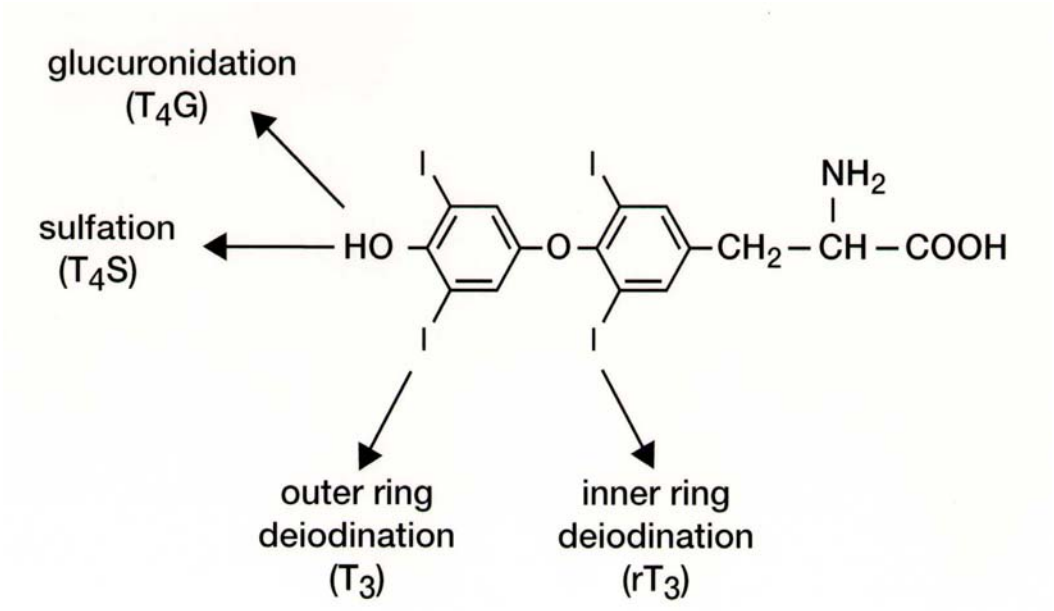

Fig. 3. Major pathways of thyroxine metabolism

Deiodination is the most important metabolic pathway. Thyroid hormone is produced in the thyroid mainly as the biologically inactive precursor T4. The daily secretion of the normal thyroid gland is about 100 nmol of T4, and about 9 nmol of T3, and less than 5 nmol metabolically inactive reverse T3 (rT3) (7). T3 differs from T4 in that it lacks an iodine atom at the phenol ring. With regard to the position of the iodine it is important to recognize that, due to the free rotation around the ether bridge, the 5 or 3 positions of the tyrosyl ring are equivalent, and also the 3' or 5' positions of the phenol ring are equivalent (Fig. 4) (108). Thus, T4 is converted by phenolic ring deiodination (outer ring deiodination, ORD) to the bioactive hormone T3; bioactive, because it is the principal ligand for the nuclear thyroid

hormone receptors (109), or by IRD to the inactive metabolite rT3. T3 is inactivated by IRD to 3,3'-diiodothyronine (3,3'-T2), a metabolite that is also generated by ORD of rT3 (36). Three enzymes are involved in thyroid hormone deiodination and these enzymes constitute a family of selenoproteins that have been highly conserved in terms of structure and function throughout vertebrate evolution. All 3 enzymes have a selenocysteine in the active site encoded by a UGA codon. However, since UGA is recognized in most mRNAs as a STOP codon, additional information in the 3'-untranslated region (3'-UTR) is necessary for selenocysteine incorporation at UGA codons. This information is presnt in the selenocysteine insertion element (SECIS-element) (110). In the following sections the characteristics of the deiodinases will be discussed (Table 2).

Type I deiodinase (D1) is expressed mainly in liver, kidney, and thyroid. Among the nonsulfated conjugates, rT3 is by far the preferred substrate, the ORD of which is orders of magnitude faster than the deiodination of any other iodothyronine (36). Therefore, it is not surprising that D1 is probably the primary site for the clearance of plasma rT3. Although it catalyzes the conversion of T4 to T3 much less effectively, D1 is supposed to be the major source of circulating T3 (111-113). The conjugated compounds T4 sulfate and T3 sulfate are preferred substrates for IRD (114). Dithiothreitol (DTT) is the in vitro cofactor and D1-catalyzed deiodination is sensitive to inhibition by PTU (115). Thyroid hormone-induced stimulation of D1 activity is exerted at the transcriptional level (116, 117), which in the human Dio1 gene can be attributed to the presence of 2 thyroid hormone response elements (TREs) in the 5' flanking region (FR) of the gene (118, 119).

Type II deiodinase (D2) is an obligate ORD and the preferred substrate of D2 is T4. Unlike D1, the role of D2 is in local T3 production. Hypothyroidism increases and hyperthyroidism decreases D2 activity. Regulation is predominantly posttranslational through substrate-induced enzyme inactivation, involving ubiquitination and degradation in the proteasome. D2 activity is expressed in pituitary, brain, and brown adipose tissue. D2 is particularly important in the brain, where it produces more than 75% of the nuclear T3 in the cerebral cortex in the rat (120). As will be discussed later for the brain, nuclear T3 in neurons is derived in a paracrine fashion from D2 activity of another cell type, indicating that local T3 production is not equivalent to an autocrine action of D2. The observation of D2 activity in skeletal muscle and thyroid gland provides new insights in thyroid hormone homeostasis (121, 122). D2 activity in skeletal muscle could serve as a source of extrathyroidally generated plasma T3 (122). Furthermore, thyroidal D2 activity in patients with Graves' disease and follicular adenomas may give rise to the relative increase in thyroidal T3

production seen in these cases as well as in iodine deficiency (121). While D2 mRNA was detected in human heart, no D2 activity was found (122). However, in a recent paper D2 mRNA as well as D2 activity were reported to be present in mouse and rat heart with an increase in D2 mRNA expression and/or activity in hypothyroidism (123). D2 mRNA or activity are present in human pituitary and brain tumors (124, 125) and in mesothelioma cell lines (126).

Type III deiodinase (D3) is an obligate IRD with T3 as the preferred substrate. It is the major T3 and T4 inactivating deiodinase, catalyzing their conversion to 3,3'-T2 and to rT3, respectively. D3 is expressed in placenta, pregnant uterus, brain, human embryonic liver, and infantile hepatic and cutaneous human hemangiomas and adult vascular tumor (127-132). D3 activity in the latter two was so high, that the inactivation rate of thyroid hormone by D3 in the tumor exceeded the secretory capacity even of the TSH-stimulated normal thyroid gland, resulting in a hypothyroidism. This clinical picture was referred to as consumptive hypothyroidism after its nature of origin. D3 activity was also detected in human brain tumors, human colon carcinoma cells and monkey hepatocarcinoma cells (133-135). Analogous to its presence in fetal as well as malignant tissues D3 is referred to as an oncofetal enzyme. Its main function in thyroid hormone homeostasis is to protect tissues from an excess of active hormone. Regulation of D3 is less obvious: whereas T3 positively regulates D3 activity at the transcriptional level in brain no regulation could be observed in placenta. This indicates that this gene is differently responsive to T3 in different tissues (108, 136, 137).

Because of differences between calculated and measured molecular mass of D1 and D2 some authors suggested that D1 and D2 are present in multimeric forms (138-141). This could mean that homodimerization or association with other proteins is necessary for catalytic activity. However, association with other cellular proteins could also regulate half-life, transport or subcellular localization of the enzyme. In a recent paper it was shown that overexpressed selenodeiodinases can homodimerize but the effect of such a dimer on deiodinase activity has not been established yet (142).

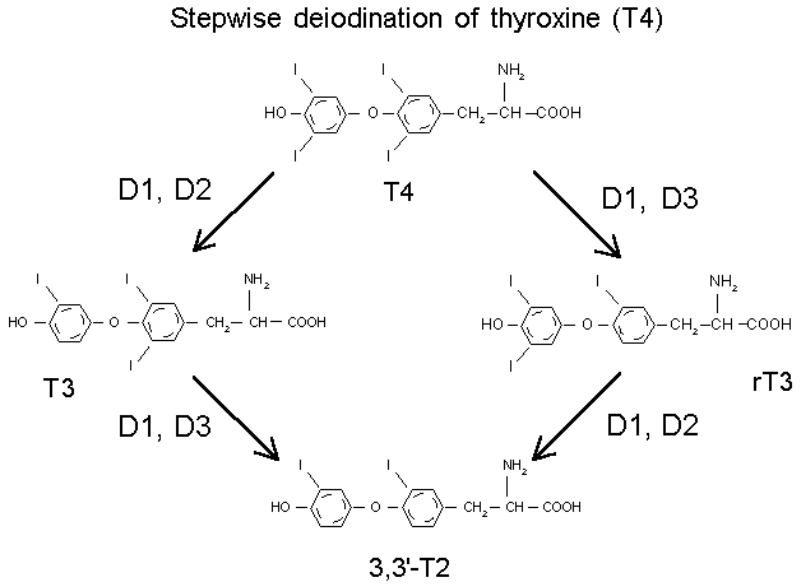

Fig. 4. Stepwise deiodination of thryoxine (T4).

Table 2. Characteristics of the human deiodinases (D1-D3) (108).

	D1 (ORD & IRD)	**D2 (ORD)**	**D3 (IRD)**
Physiological role	Plasma T3 production; rT3 and T3S degradation	Intracellular T3 & plasma T3 production	T3 & T4 inactivation
Tissue location (activity)	Liver, kidney, thyroid, pituitary	CNS, pituitary, placenta, thyroid, skeletal muscle	Placenta, CNS, fetal liver, hemangiomas
Subcellular localization	Plasma membrane (kidney and thyroid); ER (liver)	ER	?
Molecular mass of monomer (Da)	29,000	30,500	31,500
Preferred substrates	rT3 (ORD); sulphate conjugates (IRD)	T4	T3
Km (M)	10^{-7}	10^{-9}	10^{-9}
Regulation by increased TH	Transcriptional + ve	Posttranslational - ve (ubiquitination)	Transcriptional + ve
Susceptibility to Inhibitors: - PTU - goldthioglucose (GTG) - Iodoacetate (IAc) - Iopanoic Acid (IOP)	 High Very high High High	 Very low High Low High	 Very low High Low High
Specific labeling with BrAcT3, T4	Yes Competitive vs T3/T4	No	Weak Competitive vs T3/T4

THE DEIODINASES IN PHYSIOLOGY

The critical role of D2 in feedback regulation on the hypothalamus

In the feedback mechanism of the hypothalamic-pituitary-thyroid axis physiological levels of T4 as well as T3 are required to suppress TRH mRNA in the paraventricular nucleus of the hypothalamus (143-146). The presence of D2 can account for the requirement for physiological levels of both T4 and T3 for normalization of TSH. Surprisingly, there is no D2 activity in the paraventicular nucleus of the hypothalamus as one would expect, but instead in the arcuate nucleus and median eminence (147, 148). Subsequent *in situ*-hybridization studies and, recently, a study using light and electron microscopy have shown that D2 is localized in the tanycytes, a specialized type of glial cells (149, 150), whereas throughout the brain D2 is mainly expressed in another type of glial cells, i.e. astrocytes. Tanycytes line the walls of the lower third and the floor of the third ventricle and extend long processes to the adjacent hypothalamus and the median eminence. Within these locations, the tanycyte processes end in capillaries and axon terminals. Expression of D2 in the tanycytes suggests that these cells are involved in the uptake of T4 from the capillaries of the median eminence and basal hypothalamus and/or from the cerebrospinal fluid (CSF), and its subsequent conversion to T3. Furthermore, identification of D2 activity in astrocytes and tanycytes supports the hypothesis that these cells play an important role in the mediation of peripheral signals, such as thyroid hormones, on hypothalamic functions (151). As discussed before, D2 is negatively regulated by thyroid hormone. An unexpected observation is that, in X. laevis tadpoles, there is an induction of D2 in the pituitary thyrotroph by T4 and T3 at metamorphic climax (152). This paradox leads to a marked reduction of TSHβ and a fall in circulating TSH at the completion of metamorphosis. Interestingly, this increase only occurs in thyrotrophs. The timing and the remarkable specificity of D2 expression in the thyrotrophs of the anterior pituitary coupled with the requirement for locally synthesized T3 to suppress TSHβ gene expression strongly support a role for D2 in the onset of the negative feedback loop at the climax of metamorphosis in X. laevis (152). The role of D2 in the negative feedback regulation of TSH secretion has recently been confirmed in D2 knock-out mice, which show elevated serum T4 and TSH levels and normal T3 levels (153).

T3 homeostasis in euthyroidism: plasma and local T3

Plasma T3 is produced by 2 different processes, namely direct thyroidal secretion of T3 or extrathyroidal ORD of T4. As mentioned before, the thyroid mainly secretes T4 and the bulk of the daily T3 production occurs in various extrathyroidal tissues via ORD of T4 by D1 and D2. Earlier studies showed that D1-catalyzed T3 production is the major component of extrathyroidal T3 production in hyperthyroidism, but this is less obvious for the euthyroid state (154-156). On the other hand, there is a significant increase in the fractional conversion of T4 to T3 in both hypothyroidism and hypothyroxinemia with an overall fractional conversion of T4 to T3 in the hypothyroid patient of approximately 50%, vs. 25% in euthyroidism (157). This is considered as a typical feature of ORD by D2, because the opposite would be expected for D1-catalyzed conversion (157-159). Depending on the assumptions used, one can obtain estimates suggesting that as much as 81% or as little as 15% of T3 derives from rapidly equilibrating (D1-containing) tissues in euthyroidism, with the remaining coming from slowly equilibrating (D2-containing) tissues (108). These results imply that ORD of T4 by D2 is a potential source of extrathyroidal T3 in euthyroid humans as well as in rats but also that an increase in D2 is an important mechanism to preserve T3 production in primary hypothyroidism (122, 160, 161).

Whereas the liver is the important site for plasma T3 production via ORD of T4 by D1, important sites for ORD by D2 are skeletal muscle and the thyroid; in particular expression of D2 activity has been detected at high levels in the thyroid (121, 122, 162). Whereas the source of intracellular T3 tissues like the liver and the kidney is plasma T3, there is a significant contribution of D2 to local T3 generation in tissues like the brain (108).

Embryonic development and maternal-fetal physiology

The thyroid hormone levels during embryogenesis are critically important. Thyroid hormone primarily promotes differentiation and thus attenuates proliferation (108). Both insufficient and elevated levels of thyroid hormone can be detrimental and result in abnormal development (8). Until 10-12 weeks the human fetal pituitary-thyroid axis is not functional, and the fetus does not synthesize thyroid hormone. However, since human fetuses have fetal thyroid hormone-occupied TR before the fetal thyroid becomes functional there must be a maternal-fetal transfer of thyroid hormones (163). Even after the onset of fetal thyroid function this transfer may still contribute to the maintenance of fetal thyroid status (108). Compared with adults, plasma T3 concentrations in the human fetus are decreased, whereas rT3 levels and the levels of the different iodothyronine sulfates, i.e. T4S, T3S, rT3S and 3,3'-

T2S, are increased (164) (Table 3). The reason for the high concentrations of sulfated iodothyronines in fetal plasma is still unknown. It is clear from these data that the pattern of circulating iodothyronines in the fetus is characterized by low levels of T3 and a high rT3 due to the combination of high D3 expression in fetal tissues such as liver and brain, the placenta, and probably the uterus, in combination with still incomplete expression of hepatic D1 throughout most of gestation (127, 165-167). In a recent study, Kester et al. showed a developmental stage-dependent and tissue-specific expression of D3 in the developing human brain, whch was strongly correlated with local thyroid hormone levels in the different brain regions (168). They also showed different temporal patterns of D2 expression in the various brain areas important for local T3 production. These data suggest important roles of both enzymes in controlling local T3 levels in the developing brain.

The placenta is the site for maternal-fetal thyroid hormone transfer and can be an important determinant of the thyroid state of the fetus (164). The presence of D3 at maternal-fetal interfaces is consistent with its role in modulating the thyroid status of the human fetus and its expression in endometrium suggests that local regulation of thyroid status is important in implantation (169). Besides D3 placenta also contains D2. However, activity is less and cellular localization is different between D2 and D3. Nevertheless, fluctuations in D2 activity could play a role in the regulation of intraplacental T3 generation. The elevated activity of D3 in the uterus, amnion and placenta represents an effective barrier to the passage of maternal thyroid hormone. Despite T4 and T3 inactivation in the placenta and uterus, neonates with congenital hypothyroidism often have little evidence of the condition at birth, suggesting significant placental transfer of maternal T3 and T4. It has been directly demonstrated that cord blood T4 levels in neonates with a total organification defect are 20-50% of normal and these decrease rapidly after birth (170). Even in severely hypothyroid newborns with markedly reduced serum T4 levels, serum T3 and placental D3 activities were similar to those of euthyroid newborns. This suggests that placental D3 is regulated by serum T3 (170). These results indicate that a steep maternal-fetal gradient somehow overcomes the placental barrier, permitting maternal T4 to enter the fetal circulation.

Table 3. Iodothyronine concentrations in maternal and fetal serum and amniotic fluid (nmol/l).

Iodothyronine	Maternal serum	Amniotic fluid		Fetal serum	
		20-wk	Term	20-wk	Term
T4	154.4	3.2	7.3	39.9	141.6
T3	3.07	0.13	0.10	0.20	0.75
3,3' T2	0.04	0.11	0.12	-	0.21
rT3	0.37	2.00	1.06	3.84	4.15
T4S	0.02	0.33	-	-	0.25
T3S	0.04	0.09	-	0.09	0.16
rT3S	0.05	0.12	-	-	0.68

Dashes indicate that data are not available. Maternal values are for midgestation. Data are derived from ref. (164)

THE DEIODINASES IN NONTHYROIDAL ILLNESS

The initial response to acute insults such as illness or trauma results in an increased availability of glucose, amino acids and free fatty acids. These metabolites are directed towards vital organs such as the brain and the immune system. This acute metabolic response is thought to be evoked partly by endocrine changes including an activated hypothalamic-pituitary-adrenocortical axis, hypersecretion of PRL and GH in the presence of low insulin-like growth factor I (IGF-I), and a low activity state of the thyroid and the gonadal axis (171). With regard to the changes observed in the pituitary-thyroid axis, most prominent are the low T3 and elevated rT3 levels, usually referred to as 'low T3 syndrome' (172, 173). Also the terms nonthyroidal illness (NTI) or 'sick euthyroid syndrome' are used (173). Since the early description of this syndrome in ill patients (174-176), low serum T3 levels have also been found in more specific cases like patients with liver disease (176-179), after stress or surgery (176, 180), in patients with chronic renal failure (176, 181), in the elderly sick (182), after the ingestion of a number of drugs (173), and also in patients with brain injuries and brain tumors (179). Patients with congestive heart failure also showed a NTI, the extent of which appeared to correlate well with disease severity (183-185). Of course, these changes have to be attributed to the illness only in the absence of an underlying disorder of the hypothalamic-pituitary-thyroid axis, and their complete reversal must accompany recovery from the causal illness (173).

Recently it was reported, that the low-T3 syndrome is a strong predictor of death in cardiac patients and might therefore be directly implicated in the poor prognosis of cardiac patients (186). The magnitude of the changes in serum T4 and T3 levels in NTI seems not to be dependent on the type of illness, but on the severity (173, 187). The most frequent change is a reduction in serum T3, occurring within 24 hours after onset of illness, with lower values associated with more severe forms of illness (172, 187, 188). The lowered serum T3 is often, but not always, accompanied by elevated rT3 concentrations. Patients with renal disorders (189, 190), with traumatic brain injury (191) and patients with acquired immunodeficiency syndrome (AIDS) or AIDS related complex (192) do not show a concomitant rise in serum rT3.

In chronic illness the hypothalamic-pituitary axis is depressed, while in acute illness this is not an important feature (171). Kinetic studies on the turnover of the hormones in patients with the sick euthyroid syndrome have revealed that the production of T3 is decreased, but its clearance is unchanged, whereas production of rT3 is unchanged, while its clearance is diminished (188, 193). This can be explained by a reduction of D1 activity in the liver during NTI, resulting in decreased peripheral T3 production from T4 and reduced breakdown of rT3 (188, 194). Recent papers also reported increased D3 activity in liver and skeletal muscle of critically ill premature infants and critically ill patients (194, 195). A stimulated D3 activity gives rise to an increased T3 breakdown as well as an increase in rT3 production out of T4. This also fits well with regard to lowered serum T3 and increased rT3 levels observed during NTI. An impairment of T4 and rT3 transport into the liver, as seen before in non-uraemic critical illness (196), could be an additional mechanism. This would lead to a reduced substrate availability for D1, and that would lead to a reduced T3 production and breakdown of rT3 (197, 198).

THYROID HORMONE AND THE CARDIOVASCULAR SYSTEM

It is well recognized that cardiovascular manifestations are some of the most profound and reproducible clinical findings associated with thyroid disease (199). The earliest descriptions of thyrotoxicosis included references to the rapid and occasionally irregular heart rate, the hyperdynamic precordium, warm skin, bounding pulses, and exercise limitations imposed by dyspnea (200-202). The magnitude of these cardiac-related findings led early observers to postulate wrongly that thyrotoxicosis was a disease originating within the heart (202). The increased blood flow to the thyroid gland that was known to occur was presumed

to be a secondary response. Only later was the role of the thyroid gland and its ability to alter thyroid hormone production recognized as the cause of the disease now well known as hyperthyroidism (202-206).

Thyroid hormone has major effects on the cardiovascular system. Cardiac functions such as heart rate, cardiac output, and systemic vascular resistance are closely linked to thyroid status. In addition to the well-recognized action of T3 to increase peripheral oxygen consumption and substrate requirements, that causes a metabolic vasodilatation leading to a secondary increase in cardiac contractility, the hormone also increases cardiac contractility directly (199, 207, 208). T3 decreases systemic vascular resistance by dilating the resistance arterioles of the peripheral circulation (209). The vasodilatation is due to a direct effect of T3 on the vascular smooth-muscle cells that promotes relaxation (210).

As a result of the decrease in systemic vascular resistance, the effective arterial filling volume falls, causing an increase in renin release and activation of the angiotensin-aldosterone axis. This, in turn, stimulates the renal sodium reabsorption, leading to an increase in plasma volume. Thyroid hormone also stimulates erythropoietine secretion. The effect of these two actions leads to an increase in cardiac output.

To understand the actions of thyroid hormone on the cardiovascular system it is necessary to review the mechanisms by which thyroid hormone acts on cardiac myocytes and smooth muscle cells (199, 207, 210, 211). As in other cells, in cardiac myocytes and smooth muscle cells T3 is the active cellular form of thyroid hormone. There is evidence for the existence of a transport protein for T3 in cardiomyocytes (90). Both MCT8 and fatty acid translocases (FAT) could be important also for transport of other iodothyronines in cardiac myocytes besides T3 (93, 101). With regard to local thyroid hormone metabolism in cardiomyocytes low D1 activity has been identified in the rodent heart (212), while D2 activity regulated by thyroid status in mouse and rat heart has been described recently (123). However, in the human heart, D2 mRNA was identified without D2 activity (122). Although on the basis of one of the studies in rodent heart local conversion of T4 to T3 could be of importance, this item is enigmatic, since D2 activity could not be identified in other species like the human and porcine heart, not even in the hypothyroid pig (Wassen FWJS, personal observation). Furthermore, since D2 mRNA and activity have been demonstrated in human aortic smooth muscle cells (hASMCs) it could well be, that the amount of D2 mRNA measured in the heart is originating from these hASMCs (213). Finally, although till now nothing is known about D3 activity in cardiomyocytes, we were able to detect D3 activity in fetal human heart (Visser TJ et al., unpublished observation).

Once T3 enters the nucleus it binds to the T3 nuclear receptors (TRs) that are members of the nuclear receptor superfamily and possess the conserved protein domain structure present in all family members. These include a variable N-terminal domain, a DNA binding domain (DBD) followed by a hinge region, and a C-terminal ligand binding, dimerization, and transactivation domain (LBD) (214) (Fig. 5). Two genes, THRA and THRB, encode the TRα and TRβ isoforms in most vertebrates. Where TRα1, TRβ1 and TRβ2 are fully functional receptors, TRα2 fails to bind hormone and acts as a weak antagonist in vitro. TRs bind to specific DNA sequences, the thyroid hormone response elements (TRE), on target genes.

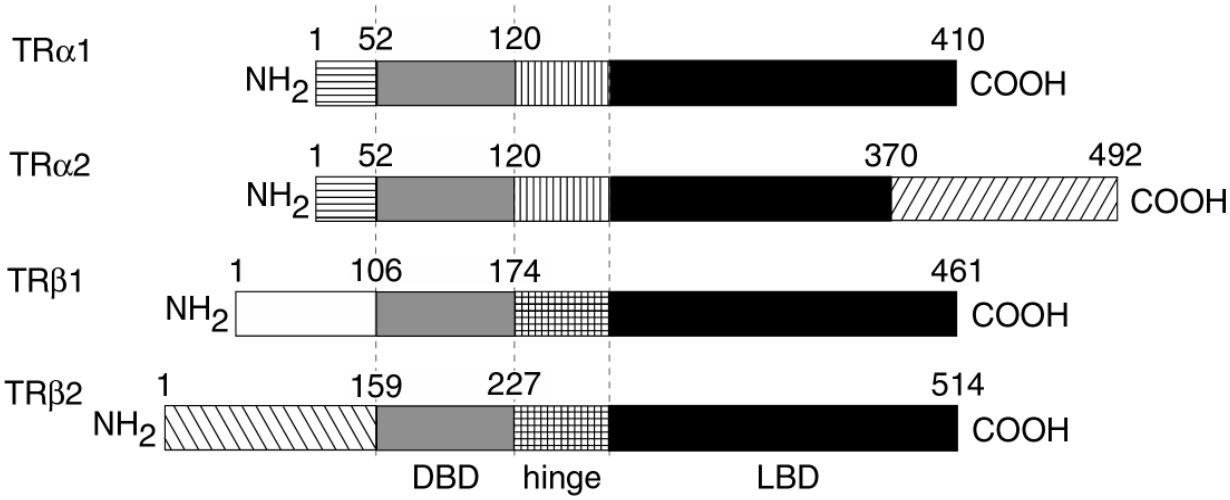

Fig. 5. The T3 nuclear receptors (TRs)

The T3 nuclear receptor complex can bind to DNA as a monomer or homodimer, but preferentially as heterodimer with members of the retinoid X receptor (RXR) family. On positive TREs, regulation is as follows. Unliganded TRs associate with co-repressors, such as SMRT (silencing mediator of retinoid and thyroid hormone receptor) or NCoR (nuclear receptor co-repressor), to repress transcription. After binding of T3 to the TR-RXR hetero-dimer, the co-repressor complex is replaced by a co-activator complex that enhances the hormone-dependent transcriptional activity of TR (Fig. 6).

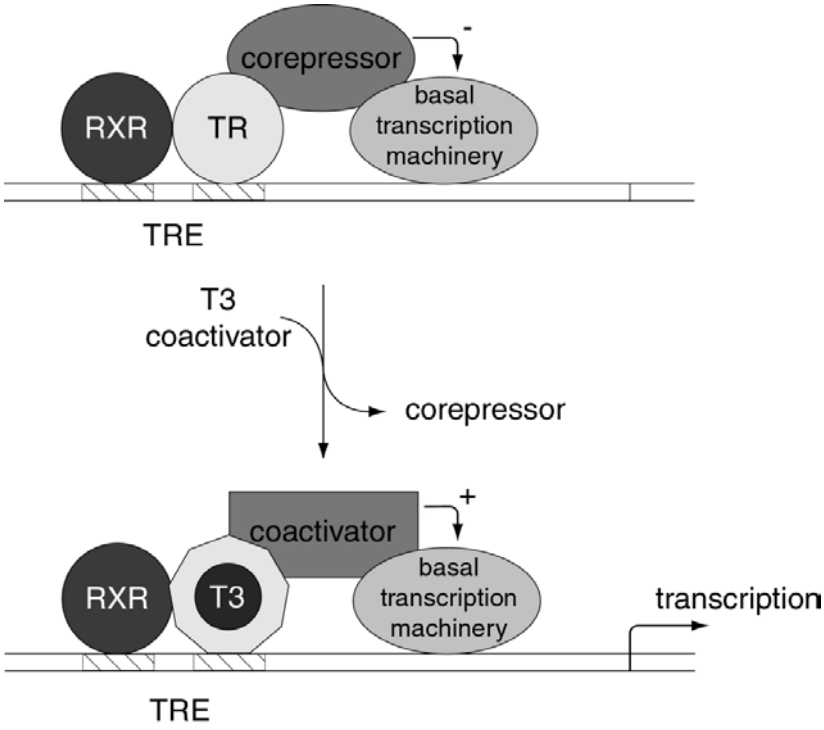

Fig. 6. Model for activation and repression of the gene expression by TR. In the absence of T3, TR/RXR recruits a corepressor complex, whereas in the presence of T3, TR/RXR/T3 releases the corepressor complex and recruits a coactivator complex

TR can inhibit or enhance gene expression depending on the nature of the TREs, availability of ligand, and cellular environment. Where cardiac genes like α-myosin heavy chain, sarcoplasmic reticulum Ca-ATPase (SERCA) and Na/K-ATPase are under positive control, cardiac genes like β-myosin heavy chain and phospholamban are negatively regulated. Release of calcium and its reuptake into the sarcoplasmic reticulum are critical determinants of systolic contractile function and diastolic relaxation (207, 215, 216). Active transport of calcium into the lumen of the sarcoplasmic reticulum by SERCA is regulated by phospholamban, activity of which, in turn, is modified by its level of phosphorylation (216). Furthermore, as mentioned before, the SERCA gene expression is stimulated by thyroid hormone, while that of phospholamban is inhibited. Thus, changes in the relative amounts of these proteins account for altered systolic and diastolic function in thyroid disease (211, 216, 217). Similarly, such changes in expression of T3-dependent Ca-handling genes have been related to contractile dysfunction in heart failure. More particular, in heart failure many T3-regulated genes show a pattern of expression akin to hypothyroidism. Therefore, a local cardiac impairment of thyroid hormone activity has been proposed to contribute to the

phenotype of heart failure. A decrease in transport, changes in metabolism or downregulation at receptor level could also be mechanisms of importance.

HEART FAILURE AND CARDIAC HYPERTROPHY

Cardiovascular disease as a whole contributed to one third of global deaths in 1999, accounting for nearly 17 million deaths that year. Coronary heart disease is the most common cause of death in Europe, accounting for approximately 2 million deaths each year. Heart failure has become the most prevalent cardiovascular syndrome, and its incidence continues to increase. Globally 7.1 million deaths are due to heart failure and this number will rise to 11.1 million in the year 2020 according to the World Health Organization (WHO). Cardiovascular diseases include diseases of the heart as well as those of the vascular system, such as acute failure due to infarction (most cases of heart failure develop as a result of myocardial infarction (218)), or arrhythmias, and chronic heart failure caused by pressure or volume overload due to continuous systemic or pulmonary hypertension, valve defects and ventricular hypertrophy as a post-infarction event. Major risk factors for the development of heart failure include coronary arteriosclerosis, hypertension, smoking, obesity, and diabetes mellitus.

It is important to realize, that heart failure is not a clearly defined disease, but it has to be regarded as a clinical syndrome with functional impairments, like decreased cardiac output and exercise intolerance (219). Attempts have been made to define heart failure based on clinical, epidemiological, pathophysiological and exercise related criteria. Interestingly, no consensus on a definition of heart failure has been reached (220). This reflects the difficulty of defining heart failure. Regardless of the origin of the pathological stimulus, congestive heart failure is almost always preceded by the development of some degree of cardiac hypertrophy. The New York Heart Association (NYHA) criteria can be used to classify the severity of the failure: Class I patients with documented heart disease of any type, who are symptom-free, till Class IV patients with symptoms at rest.

Treatment of heart failure patients is costly, the rise in life expectancy in developing countries plays an important role in this, and therefore prevention is important. Prevention can be performed by giving general advice about a healthier life-style and by more specific measures like correction of high serum cholesterol levels and good control of diabetes and hypertension. Mortality increases from classes I to II of the NYHA categories and sharply increases from class III to IV.

Sustained increase in workload due to, for instance, hypertension, loss of viable tissue or valve defects, causes the heart to respond with the development of hypertrophy. This response may be successfully compensatory, but if the pathological stimulus is maintained over a prolonged period of time it will eventually progress into heart failure. In contrast to hyperplasia, where the number of cells increases, the augmentation in cardiac mass during the development of hypertrophy is a process in which cells increase in size instead of number. This requires the activation of growth related genes, involving the reappearance of a fetal gene program (221-224) and an increase in protein synthesis (225). Other characteristic events are the activation of proto-oncogens, such as c-fos, c-jun and c-myc (226), a reduction in myocardial fatty acid oxidation and increased glucose utilization (227), a desensitization of β-adrenergic receptors caused by chronically elevated norepinephrine levels (228, 229), a decrease in circulating thyroid hormone levels (211) and an increase in inflammatory cytokines (230, 231).

Cardiac hypertrophy can be the result of two distinguishable, but often simultaneously occurring pathological stimuli. Volume overload causes excentric hypertrophy due to elongation of cells and the alignment of newly formed contractile units in series. In contrast, pressure overload induces concentric hypertrophy where the diameter of the cardiomyocyte is increased and additional contractile units are formed in parallel. Mechanically, the development of cardiac concentric hypertrophy normalizes the wall stress, thus relieving the pathological stimulus, enabling normal ventricular function. Yet, the only known forms of hypertrophy not progressing into heart failure are those caused by intense training, as seen in athletes, or by mild hyperthyroidism (232). Hypertrophy generally precedes heart failure, but can be sustained over variable periods of time, before heart failure develops. Nonetheless, cardiac hypertrophy itself is an independent risk for sudden death caused by arrhythmias and ischaemic heart disease (225). The processes underlying the onset of progression of cardiac hypertrophy into heart failure are poorly understood and a better knowledge and the ability to control these events would offer the possibility to reverse the process or at least prevent the transition and thereby reduce morbidity and mortality.

The accepted standard medical treatment for severe heart failure includes therapy with ACE inhibitors (angiotensin-converting-enzyme inhibitors), digoxin and diuretics. Large clinical trials have shown that ACE inhibitors provide symptomatic benefit in most patients and reduce mortality by 18% to 30% in all degrees of symptomatic heart failure.(233, 234). They act by preventing the body from producing angiotensin, a substance in the blood that causes vasoconstriction, stimulates production of aldosterone, and raises blood pressure.

Diuretics are prescribed for almost all patients who are experiencing fluid buildup in the body and swelling in the tissues. A diuretic causes the kidneys to remove more sodium and water from the bloodstream than usual. Since there is less fluid to pump throughout the body, the workload of the heart is relieved. It also decreases the buildup of fluid in the lungs, the legs and the ankles. Nitrates cause a preload reduction and have a complementary role to diuretics. Digoxin increases the force of the heart's contractions, which can be beneficial in heart failure. This relieves heart failure symptoms, especially in cases where the patient is not responding to ACE inhibitors and diuretics. It also slows certain types of irregular heartbeats. The heart tries to compensate for its weakened pumping action by beating faster, which puts more strain on it. Beta-blockers (β-blockers) reduce the heart's tendency to beat faster. This allows the heart to maintain a slower rate and lowers blood pressure. Beta-blockers are used for mild to moderate heart failure and often with other drugs such as diuretics, ACE inhibitors and digoxin. People with heart failure are at risk of developing blood clots, usually in the blood vessels of the legs, lungs and the heart. The last type can occur in cases of a condition called atrial fibrillation, which happens when the chambers (atria) of the heart contract rapidly in an uncoordinated fashion. This causes the blood to pool in the atria, where it can form clots. These can be propelled into the blood vessels that supply the brain. If one gets stuck in a vessel, blood flow to the brain is cut off and stroke results. Patients with a history of clots in the lungs or legs, atrial fibrillation or stroke will get anticoagulant therapy in order to prevent strokes. To prevent venous thrombosis stockings and leg exercise can be prescribed as well.

Amiodarone is a benzofuranic-derivative iodine-rich drug, widely used for the treatment of tachyarrhythmias, i.e. ventricular arrhythmias, paroxysmal supraventricular tachycardia, and atrial fibrillation and flutter (235), and to a lesser extent also employed for severe congestive heart failure because of its minimal negative inotropic action (236). A well known, yet indirect, effect of amiodarone is its inhibitory action on liver D1 activity. This results in decreased peripheral conversion of T4 to T3 (173, 237-241) and there is a concomitant increase in serum rT3 levels due to its decreased clearance. In addition, the drug inhibits thyroid hormone entry into peripheral tissues, i.e. mainly the liver (242). Both mechanisms contribute to the increased serum T4 concentration and the decreased serum T3 concentration in euthyroid subjects given long-term amiodarone therapy (243-246). This increase is found as early as 2 weeks after institution of amiodarone therapy (237, 247). The increase in rT3 is usually far greater than the decrease in serum T3 concentrations (248, 249). Indeed, serum T3 concentrations often remain within the low normal range (250). Amiodarone administration is also associated with dose- and time-dependent changes in serum TSH concentration. With a

daily dose of 200-400 mg of the drug, serum TSH levels are usually normal, although an increased TSH response to intravenous TRH administration is frequently observed (245). With higher doses of the drug an increase in serum TSH may occur during the early months of treatment, but this is generally followed by a return to normal (237, 247). The changes in serum TSH concentration could be attributed to the direct effect of amiodarone on TSH synthesis and secretion at the pituitary level (250). The increased serum TSH concentration may also result from the inhibition of pituitary D2 ORD activity by either amiodarone or desethylamiodarone, thereby preventing local T3 production resulting in a decreased intrapituitary concentration of T3 (251). Indeed, after a loading dose of amiodarone by intravenous infusion, TSH is the first hormone to undergo significant variations, i.e. it increases, even during the first days of therapy (252). During long-term amiodarone therapy, clinically euthyroid patients may show modest increases or decreases in serum TSH concentration, possibly reflecting episodes of subclinical hypo- or hyperthyroidism, respectively (250). Altogether, thyroid function tests are often changed under amiodarone therapy, showing typically an increase in serum T4 and rT3, and a decrease in serum T3 in clinically euthyroid patients. However, in 14-18% of amiodarone-treated patients, there is overt thyroid dysfunction, either amiodarone-induced thyrotoxicosis or amiodarone-induced hypothyroidism (250). The latter condition is caused by the large amount of iodide liberated from amiodarone. In addition to the effects on thyroid hormone transport and metabolism, amiodarone also inhibits T3 action at the receptor level (253, 254)

STRUCTURE-FUNCTION RELATIONSHIP OF DEIODINASES

Iodothyronines and deiodination: an historical perspective

Iodine was first identified in the thyroid gland 100 years ago by Baumann (255), who also demonstrated that most of it was contained in a protein fraction that, on hydrolysis, yielded a substance that was effective in reversing both symptoms of myxedema in women, and the effects of thyroidectomy in animals (256). Chemical purification followed in 1914, when Kendall crystallized a compound containing 65% iodine that he called thyroxine (257). Twelve years later Harington succeeded in characterizing (258, 259) and synthesizing (260) thyroxine. At that time it was assumed that thyroxine was the only thyroid hormone.

This view was held until 1951 when the first evidence that there might be a second hormone was obtained; Gross and Leblond (261), using radioactive isotopes and paper chromatography, reported the presence of a radioactive compound different from T4 in the

plasma of rats given ^{131}I. Gross and Pitt-Rivers (262) also found this compound in the plasma of humans treated with ^{131}I and demonstrated that it was 3,5,3'-triiodothyronine (T3), the ORD-derivative of T4, and that it possessed thyromimetic activity (263). They concluded that T3 was the peripheral thyroid hormone and T4 was its precursor, based on the fact that T3 was considerably more potent than T4 (264), and that the thyroid contained much less T3 than T4 (265). Although at that time no direct evidence existed, the presence of a system for the ORD of T4 is implicit in this conclusion.

It was not until 1970, when the development of binding-displacement techniques permitted the measurement of very low levels of thyroid hormones in plasma (266), that Braverman et al. (267) demonstrated unequivocally the presence of T3 in the plasma of athyreotic subjects following administration of T4, thereby clearly implicating the existence of an ORD in peripheral tissues. This was followed by a plethora of studies in both humans and animals that established that much of the T3 in plasma is produced by peripheral deiodination of T4 (193), and that the principal ligand for the nuclear receptor is T3 (109).

Work over the subsequent two decades consisted primarily of documenting the presence of two different enzyme activities that catalyzed T4 to T3 conversion. Based on kinetic analyses and patterns of inhibition by compounds such as PTU and GTG, two separate enzymes possessing ORD activity have been identified in mammalian tissues (268-270) and designated the types 1 and 2 deiodinases (D1 and D2 respectively). Furthermore, a third deiodinase, the type 3 (D3), was identified and this enzyme catalyzes the IRD of T4 and T3 to rT3 and T2, respectively, compounds believed to have minimal activity. This makes D3 an inactivating deiodinase. Although it has been shown that D1 can catalyze IRD in addition to ORD (268), the existence of D3 as a separate enzyme has now been clearly established based on enzymatic properties (271-274) and from molecular cloning studies.

Given the unavailability in the late 1980s of antibodies, probes, or structural information concerning the deiodinases, expression cloning strategies were utilized, whereby a cDNA for a deiodinase could be identified on the basis of the functional activity of its protein product (114). An important advance was the demonstration that X. laevis oocytes could be induced to express D1 activity when injected with polyadenylate (poly A^+) RNA isolated from the liver of a hyperthyroid rat providing a functional assay for screening appropriate cDNA libraries (275). Berry et al. succeeded in isolating a 2.1-kb full-length D1 cDNA (designated G21) using a strategy that depended on the direct induction of D1 activity in oocytes (116). G21 contained at codon 126 an in-frame TGA triplet that theoretically could be a termination codon, but in this protein it codes for the rare amino acid selenocysteine (Sec) situated in the

catalytic site of D1 (116). This finding confirmed prior reports of D1 being a selenoprotein (276, 277). Actually, D1 was only the second eukaryotic mRNA shown to encode a selenoprotein, the first being glutathione peroxidase (GPX).

Subsequent studies led to the cloning of type 3 iodothyronine deiodinase (D3) by Wang and Brown (278), isolated as a 1.5 kb cDNA from X. laevis tadpole tail tissue and designated as XL-15. This was first recognized as a highly T3-responsive cDNA with similarity to mammalian D1 cDNA, including the presence of an in frame TGA triplet. Although it was assumed on forehand that the XL-15 represented an amphibian equivalent of the mammalian D1, expression of the XL-15 in X.Laevis oocytes yielded a deiodinase with properties characteristic of a D3, i.e. IRD activity and resistance to inhibition by PTU (279). Thereafter, D3 cDNAs from rat (280), Rana catesbeiana (R. catesbeiana) (281), human (282), chicken (283), and tilapia (284) were isolated.

With regard to the cloning of D2 it was assumed that D2 could be a third member of the deiodinase family of selenoproteins. Therefore, comparison of the D1 and D3 cDNAs revealed three short but highly conserved regions, one of which encompasses the TGA selenocysteine codon. These sequences were utilized as the basis for a PCR strategy that led to the cloning of a D2 cDNA from R. Catesbeiana metamorphosing tadpole tail and leg mRNA (285). Thereafter, cDNAs for the rat, human, and fish (Fundulus heteroclitus) D2 have also been identified (160, 286), whereas recently human, mouse and chicken D2 cDNAs containing intact 3' untranslated regions (3' UTR) (5-7.5 kb) were successfully identified. These D2 cDNAs encode functional proteins in X. laevis oocytes and/or transiently transfected cells (287-289). All three deiodinases contain the highly conserved region surrounding the TGA codon that forms part of the active catalytic site. Furthermore, they all share a hydrophobic amino terminus region and conserved histidines (160).

The cotranslational incorporation of Sec into the deiodinases and other selenoproteins presents significant problems for the cell. It has to recognize the UGA stop translation signal as a Sec codon. The cloning of D1 led to the identification of the eukaryotic Sec insertion sequence (SECIS) element as a stem-loop structure in the 3'-UTR of the D1 and glutathione peroxidase mRNAs (108). The SECIS element is the signal that recodes the in-frame UGA from a STOP to a Sec codon (110). From D1 studies it was shown, that spacing between the SECIS element and the UGA codon is of importance, in that a minimal spacing requirement has to be met (290). Whereas increasing the spacing between the UGA codon and SECIS element by the insertion of 1.5 kb had no effect on SECIS activity, deletions that narrowed the spacing to less than approximately 60 nucleotides (nt) abolished Sec incorporation (291). At

the other extreme, human D2 mRNA shows a spacing of 5 kb, and this is the longest separation between a Sec encoding UGA and SECIS element in any eukaryotic selenoprotein mRNA reported to date. Apparently, the distance between the UGA codon and the SECIS element may be very large (287-289).

An additional 10 years were required for the essential components of the eukaryotic selenoprotein synthesis machinery to be identified (292, 293). The *trans*-acting factors, identified in bacteria and encoded by genes designated Sec synthase (selA), elongation factor with mRNA stem-loop binding activity (selB), tRNA[Ser]Sec (selC), and selenophosphate synthase (selD) (294-299) are important in selenoprotein synthesis. Instead of one selB factor in bacteria, a heterodimer of the SECIS-binding protein 2 (SBP2) (300, 301), and the elongation factor (EFsec) are involved in Sec incorporation in archaea and mammals (292, 302, 303). From all this information a model for Sec incorporation in eukaryotes can be constructed (Fig. 7) (108). The SECIS element recruits SBP2, an event that theoretically can occur in the nucleus as soon as this region is transcribed. The SECIS-SBP2 complex could then recruit the EFsec-tRNA complex, loop back to the ribosome and deliver it in the coding region. Because the SECIS element is located in the 3'-UTR in eukaryotes and not in the coding region as in prokaryotes, it obviates the need for dissociation and reassociation of the SECIS-SBP2 complex with each incorporation cycle.

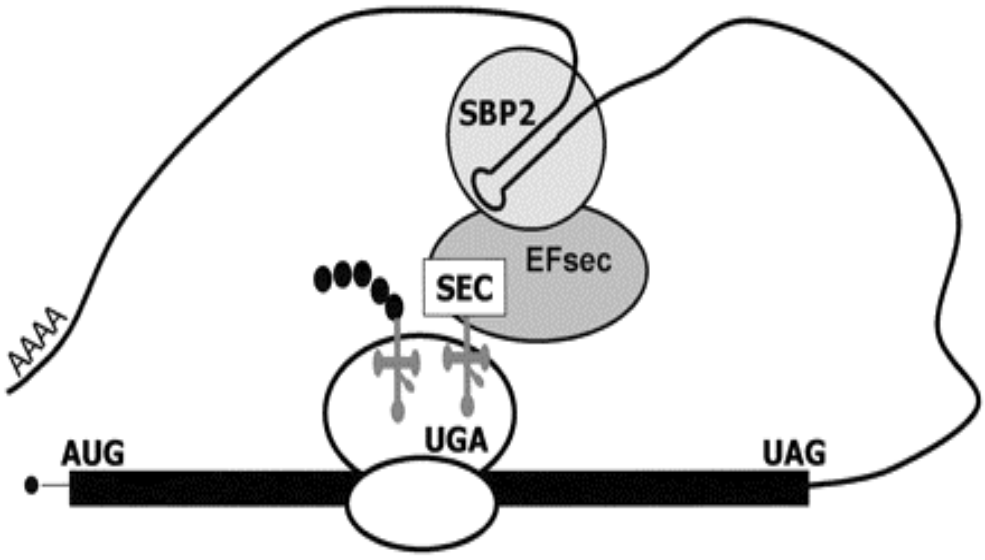

Fig. 7. Selenoprotein synthesis

Type I deiodinase (D1)

As mentioned before, D1 was the first deiodinase to be recognized by biochemical assays of T4 to T3 conversion and was also the first to be cloned. D1 is expressed in liver, kidney, thyroid, and pituitary and this enzyme is largely responsible for the production of serum T3 from T4 in euthyroid animals (304, 305). Remarkably, D1 is capable of both ORD and IRD, and shows preference for rT3 as the substrate (107, 116, 306). Interestingly, conjugation of the phenol hydroxyl with sulfate markedly enhances the suitability of the iodothyronine substrates for D1-catalyzed IRD (107, 307, 308). This is reflected in a markedly higher Vmax/Km ratio for those substrates. Actually, sulfated T4 metabolites are solely deiodinated by D1 (309). Sulfation is a critically important modification of T3 and T4 because it facilitates rapid inactivation by IRD. D1 activity *in vitro* is stimulated by thiol compounds like dithiotreitol (DTT) and a critically important characteristic of D1-catalyzed deiodination is its sensitivity to inhibition by PTU (115, 310). *N*-bromoacetyl- [^{125}I]T3 has proven to be a useful affinity label of D1, allowing the specific labeling of the 27-kDa protein in microsomal fractions (311, 312). Depending on the detergent used, the molecular mass of the solubilized wild-type enzymes is about 50-60 kDa, suggesting that the D1 protein is composed of a homodimer of 27-kDa subunits (313, 314). Since dimers containing only one wild-type partner are already catalytically active, the functional significance of dimerization remains unclear (142, 314). Although there is general agreement, that D1 is an integral membrane protein, the question whether mature D1 is a plasma membrane protein is still under study. In kidney and thyroid cells, D1 is present in the plasma membrane (314-317). Studies in rat hepatocytes suggested that in liver cells D1 is present in the endoplasmic reticulum with its active site oriented to the cytoplasm (318, 319). In transiently transfected HEK-293 cells D1 was localized in the plasma membrane as determined by immunofluorescence confocal microscopy (320). More detailed structure-function analysis became possible after cloning of the D1 cDNA (116). The complete cDNA sequences have been determined for rat, human, mouse, dog, chicken, tilapia and killifish D1 proteins (116, 283, 321-325). The mRNA sizes are about 2-2.1 kb and all contain a UGA codon in the region the active center, which is highly conserved among species (108).

Studies with both endogenous and recombinant enzymes indicate that the deiodination reaction catalyzed by D1 follows a two-step mechanism. Actually, the deiodination reaction catalyzed by D1 follows ping-pong kinetics with two substrates, the first being the iodothyronine, and the second being an unknown endogenous intracellular thiol cofactor (310, 326-330). In the first step an iodonium (I^+) ion is transferred from the iodothyronine to an

acceptor group in D1, resulting in the formation of an enzyme selenenyl iodide intermediate. In the second step this intermediate is reduced by a cofactor, DTT in *in vitro* studies, regenerating D1and yielding oxidized cofactor and iodide. PTU inhibits D1-catalyzed deiodination by competing with the putative thiol cosubstrate to form an essentially irreversible Enzyme-Se-S-PTU dead-end complex (108). In the reduced selenolate form, the selenocysteine is the target for potent inhibition by goldthioglucose (GTG) and iodoacetate (IAc) (108).

The important role of the nucleophilic properties of Se vs. S are illustrated by the roughly 100-fold lower turnover number for the Cys126 mutant of D1 relative to the Sec wild type (108, 326) while it should be noted, that the efficiency of translation of the Cys126 is 50-100 times higher than SecD1 due to the inefficiency of selenoprotein translation in eukaryotes (326). Comparing the deduced amino acid sequences of rat, human, dog, chicken, tilapia, and killifish D1 reveals that only a single domain in the N terminus is sufficiently hydrophobic to qualify as a transmembrane sequence. *In vitro* translation studies showed that the transmembrane domain of rat D1 is located between basic amino acids at positions 11 and 12 and a group of charged residues at positions 34-39 (331) (Table 4A).

Table 4A. Structure-function relationship in D1 deiodinase

Position	Residues	Analysis	Effect	Ref
45-46	Gly>Asn Glu>Arg	A,B	↑ Vmax for rT3	(323, 332)
48-52	TGMTR	A,B	No effect of deletion on rT3 deiodination	(323, 332)
65	Phe>Leu	A,B	↑ Km for rT3	(323, 332)
124	Cys>Ala	A	↑ Km for DTT;↑ Ki for PTU	(333, 334)
126	Sec>Cys	A	↓↓ Activity; ↑ translation	(326)
128	Ser>Pro	A,B	PTU insensitive, sequential kinetics	(335)
156	Glu>Ala	A	No Activity	(335)
156	Glu>Asp	A	↑ Km rT3	(335)
158	His>Gln	A	No Activity	(336)
158	His>Asn	A	No Activity	(336)
158	His>Phe	A	No Activity	(336)
163	Trp>Ala	A	No Activity	(335)
174	His>Gln	A	↑ Km for rT3; ↓ sensitivity to DEPC	(336)
174	His>Asn	A	↑↑ Km for rT3	(336)
185	His>Asn	A	No Effect	(336)
194	Cys>Ala	A	Modest ↑ Km + ↓ Vmax for rT3	(333, 334)
214	Glu>Ala	A	↑ Km rT3	(335)

The numbering of amino acid residues corresponds to the position in human D1 [gi: 3041700]. Analysis method A refers to site-directed mutagenesis studies and method B to natural occurring variants.

Comparisons of the D1 enzymes of different species together with site-directed mutagenesis have led to the recognition of structurally important amino acids (Table 4A). Comparative functional-structural analysis of dog and human D1 enzymes showed, that there was a roughly 20-fold higher Vmax/Km ratio for ORD of rT3 by human than dog D1 and that the amino acids between 30 and 70 of dog D1 accounted for the 30 fold increase in Km value for rT3 ORD as observed in dog compared to human D1 (323, 337). More detailed studies demonstrated that it is mainly the Phe65Leu substitution which explains the slow ORD of rT3 by dog D1 vs. human and rat D1 (323, 332). The Phe65 is critically important for ORD of rT3 and 3,3'-diiodothyronine sulfate (T2S) but not for deiodination of substrates with two iodines on the inner ring (323, 332). It suggests a specific interaction of the inner ring of rT3 and 3,3'-T2S with Phe65, possibly through π-π interactions of the two aromatic rings, which is permitted by the absence of the bulky I atom at position 5 (332). Dog also differs from rat and human D1, because it is missing a 5 amino acid structure, the TGMTR (residue 48-52 of human or rat D1). Insertion of TGMTR in dog D1 did not have any effect on deiodination of rT3, but there was a marked decrease in the ORD of T2S which could have represented interference with the interaction of the SO4 group with the active center (332). Taken together, the TGMTR deletion seems not to be critical to D1 function (108).

Although there is a high conservation of amino acids in the active center of D1 in various species, tilapia D1 is an exception in that proline replaces serine at position 128 yielding a PTU insensitive D1 (324). Since the PTU insensitive D2 and D3 enzymes also have a proline at this position, site-directed mutagenesis was tested to restore PTU sensitivity. However, PTU sensitivity was not restored, indicating that the explanation for the PTU insensitivity of tilapia D1 lies elsewhere in the protein sequence.

The presence of active site histidine (His) residue(s) was postulated (338). It was previously reported that mutation of His253 did not have any negative effect on rat D1 activity, demonstrating that this histidine is not essential for deiodination (110). By comparing the deduced amino acid sequences of rat and human D1 it was confirmed that His253 is not required for deiodinase activity. It was shown, that the positions of His158, 174, and 185 are conserved between the rat and human, while His253 is not present in the human enzyme, which terminates at amino acid 249 (321). Site-directed mutagenesis studies of the four His residues in rat D1 showed, that mutagenesis of His185 had no effect, while mutagenesis of His158 resulted in complete loss of activity, suggesting a role either in protein conformation or catalysis. Mutagenesis of His174 to asparagine (Asn) or glutamine (Gln) caused a significant increase in the Km for rT3 deiodination. Furthermore, mutation of His174 to Gln

greatly reduced sensitivity to diethylpyrocarbonate (DEPC) inhibition at any DTT concentration tested as DTT might protect selenocysteine from this reagent. However, residual DEPC inhibition of D1His174Gln at 1 mM DTT was not reversible by hydroxylamine, indicating that this inhibition is due to reaction of DEPC with residues other than His. Identical to what was described for DEPC, the sensitivity of the His174Gln mutant for rose bengal (tetrachlorotetraiodofluorescein) inhibition was significantly reduced with Ki of 0.5-0.7 μM as compared with 80-90 nM for the wild-type D1 (336). These results demonstrate that His174 is the major target for the His-specific inhibitors DEPC and rose bengal. This His residue is critical to D1 function and appears to be involved in binding of substrate (336).

In addition to the selenocysteine, each of the three isoforms contains 6 or 7 cysteine (Cys) residues. Of particular interest is the Cys at position 124 of the D1. This residue is vicinal to the Sec at position 126 and also present in the analogous position in D3 but not in the D2 where the corresponding amino acid is alanine (Ala). Also of interest is Cys194 in the D1, which has been conserved in all three deiodinase isoforms. The conservation of these Cys residues suggests that they may play an important role in the catalytic properties of these enzymes. In addition to acting as active site nucleophiles, Cys residues in other enzymes have a number of important functions, including a) optimal positioning of substrates within the catalytic cleft (339), b) transfer of reducing equivalents from *in vivo* reducing systems to the active site (340), and c) acting as important structural components by the formation of disulfide bridges (341) or as a result of the hydrophobic properties of the Cys side chain (342). Therefore, kinetic studies have been performed using a rat D1 enzyme in which the vicinal Cys at position 124 was replaced by the Ala found in D2 to test whether this residue is involved in catalysis by D1 (333, 334). Rat D1 Cys124Ala had a 10- to 15-fold higher apparent Michaelis-Menten constant (Km) for DTT than wild type, suggesting that the SH group of this Cys residue was involved in the interaction with the second substrate. However, the maximum velocity (Vmax) and Km for rT3 of the Cys124Ala mutant was not significantly different, although there was a 2-fold increase in the Ki for PTU. This supported a reaction mechanism for D1 in which DTT interacts with the vicinal Cys to facilitate reduction of the oxidized Se in the active center (333). In the same study Cys194 in D1 was replaced by Ala. Since this amino acid is well conserved at this position in all three deiodinase classes this is suggestive for an important role for this residue. This caused a modest increase in the Km and decrease in Vmax for rT3 (334). Neither Cys124Ala nor Cys194Ala mutations affected the rate of deiodination in transiently transfected COS-7 cells expressing these mutant D1

enzymes. Interestingly, although the Cys124Ala mutant demonstrated an increase in the Km for DTT and a decrease in Vmax in *in vitro* experiments, this can not be rate-limiting *in vivo*, since neither the Cys124 nor the Cys194 mutation affected the rate of deiodination in transiently transfected COS-7 cells (334).

Although no deiodinase mutations have been reported so far in patients, several single nucleotide polymorphisms (SNPs) have recently been described for the D1 gene that were associated with plasma TSH and iodothyronine levels in a population of 158 healthy persons (343). These SNPs are located in the 3'-UTR and yield 3 different haplotypes in this population, with haplotype 2 showing positive correlation with rT3, rT3/T4 and negatively with T3/rT3 ratios, whereas haplotype 3 was negatively correlated with rT3, rT3/T4 and positively, but not significantly, with T3/rT3. Haplotype 1 did not show any correlation with serum thyroid hormone levels. Although the SNP-related changes in thyroid hormone levels are subtle, they still can have consequences for quality of life, cognition, heart rate and other thyroid hormone directed processes and also for set points of endocrine feedback regulation (344).

Type II deiodinase (D2)

D2 activity was first identified in pituitary as a PTU-insensitive T4 ORD (345-347). Later results showed that it has a low Km for T4 (~ 5 nM), about 3 orders of magnitude lower than that of D1 under similar *in vitro* conditions (269). Reverse T3 is also an excellent substrate although less favored than T4 (122). Deiodination by D2 requires an endogenous reducing cofactor and although the identity of such a cofactor *in vivo* is unknown DTT works efficiently *in vitro*. As compared to D1, D2 activity is 100-fold less sensitive to inhibition by GTG and IAc (269, 348-350) (Table 2). Although the inhibitory action of GTG and IAc on D2 activity is relatively weak in relation to their effect on D1 activity, D2 is still highly susceptible to GTG. Furthermore, it has been shown previously, that D2, like D1 and D3, is inhibited competitively by IOP and there also is a high susceptibility for this compound (351) (Table 2).

With regard to the catalytic mechanism, though incompletely described, there is a difference between D1 and D2. Whereas ORD of T4 by D1 exhibits ping-pong (bi)-substrate kinetics with T4 and the thiol-containing cofactor as cosubstrates (269, 333, 352, 353), D2 exhibits sequential reaction kinetics, suggesting that T4 and the thiol-containing cofactor must interact with the enzyme simultaneously before reaction takes place (269, 354).

Another remarkable difference between D1 and D2 is, that where the N-bromoacetyl derivatives of T4 or T3 are excellent covalent affinity labels of D1, they do not label D2 (122, 311, 312, 355). The inability to covalently label D2 with bromoacetyl-iodothyronine derivatives as well as the drastically reduced sensitivity to PTU, IAc and GTG compared with D1, have been interpreted as evidence that deiodination by D2 does not need the participation of enzyme sulfydryl groups (356) or that D2 is not a selenoprotein (326, 357). The inability to label the "substrate-binding subunit of D2 (p29)" in rat glial cells with ^{75}Se was also interpreted as evidence that D2 is not a selenoprotein (358). However, since the cloning of D2 cDNAs from several species and the identification of SECIS-elements in the 3'-UTR, it is clear that the D2 protein is a selenoenzyme containing a Sec residue in the catalytic center (122, 160, 285-288, 359, 360). The cloning of a complete D2 cDNA was challenging due to its huge size. Human, mouse, and chicken cDNAs containing intact 3'-UTR (5-7.5 kb) were successfully identified. Human and rat D2 mRNA are approximately 7.5 kb, whereas chicken D2 mRNA is approximately 6 kb (122, 160, 287, 288).

More direct evidence for the selenoprotein character of D2 was recently provided by the demonstration that a ^{75}Se-labeled protein of the expected size is immunoprecipitated from a human mesothelioma cell line (MSTO-211H) with a D2 antiserum (126). Remarkably, substituting Cys for SeC133 causes a 1,000-fold higher Km for the substrate T4 (361, 362). It is clear that the described differences between D1 and D2 must be influenced by other factors than the presence of Sec in the catalytic center.

Comparing the core catalytic center of D1 and D2, a highly conserved region of 15 amino acids long surrounding the essential Sec, all the D2 proteins cloned so far show an Ala two residues amino-terminal to the Sec, whereas all D1 proteins contain a cysteine at this position (114, 360, 363, 364). A Cys residue in the catalytic center could assist in enzymatic action by providing a nucleophilic sulfide or by participating in redox reactions with a cofactor or enzyme residues. Site-directed mutagenesis studies were performed to investigate whether the earlier described differences between D1 and D2 could be attributed to the effect of the absence of a Cys residue in the catalytic center of D2 (Table 4B).

Whereas the D2 Ala131Cys mutant was enzymatically active with Km and Vmax for the substrates T4 and rT3 similar to those of wild type (wt) D2 the most striking finding was the reduced limiting Km for the cosubstrate DTT. Introduction of the Cys residue did not significantly increase the sensitivity of D2 for PTU, GTG or IAc (361). This suggests that the wt D2 and D2 Ala131Cys enzymes use a different mechanism of deiodination than D1. This conclusion is further supported by the fact that both the wt and D2 Ala131Cys enzymes

appear to follow sequential reaction kinetics (361), i.e. both the substrate and a thiol must combine with the enzyme before the reaction takes place (269), whereas D1 follows ping-pong kinetics (36, 269, 326, 333).

Table 4B. Structure-function relationship in D2 as studied by site-directed mutatgenesis

Position	Residues	Effect	Ref
92	Thr>Ala	No effect	(343)
128	Phe>Ala	No Effect	(335)
131	Ala>Cys	↓ Km for DTT	(361)
131	Ala>Ser	No effect	(361)
133	Sec>Cys	↑↑ Km for T4; ↑ expression	(361, 362)
133	Sec>Ala	No activity; ↑ expression	(362)
134	Pro>Ala	No Effect	(335)
135	Pro>Ser	↑ Km T4, PTU sensitive, ping-pong kinetics	(335)
136	Phe>Ala	No Activity	(335)
163	Glu>Ala	No Activity	(335)
163	Glu>Asp	No Effect	(335)
165	His>Asn	No Activity	(335)
170	Trp>Ala	No Activity	(335)
185	His>Gln	No Effect	(335)
266	Sec>Cys	Wild type activity	(359)
266	Sec>stop	Wild type activity	(359)

The numbering of amino acid residues corresponds to the position in human D2 [gi: 7549805]

A Sec133Ala exchange inactivates the D2 enzyme (362). Both the Sec133Cys and Sec133Ala human D2 mutants are expressed at levels approximately 100-fold higher than wild type, due to increased translation efficiency of the mutants (362). The sensitivity of the human D2 Sec133Cys mutant to inhibition by GTG is approximately 100-fold lower than of wt D2 (357).

After these results a model of reductive dehalogenation for D2 enzyme kinetics was proposed (361). The Sec residue exerts a nucleophilic attack to the 2'-position of T4, thus forming a bond between the Sec residue and T4. Subsequent steps involve the abstraction of iodonium (I^+) by DTT (or another thiol group containing cofactor), providing a D2·T3 complex and a cofactor sulfenyl iodide. The latter will rapidly dissociate into iodide (I^-) and oxidized thiol, whereas the enzyme intermediate will yield T3 and the unmodified enzyme. The reaction as a whole requires two thiol groups, which could be supplied by one DTT molecule. In this model, the activated substrate is directly reduced by the cofactor, whereas in the case of D1 the SeI intermediate is reduced by cofactor (269, 333, 352, 356).

Transiently expressed ^{75}Se human D2 appears as a doublet, suggesting that C-terminal UGA acts either as a STOP or as a Sec codon (122). When the second UGA of human D2 was converted into a UGC coding for Cys or for UAA, an unambiguous Stop codon, the deiodination properties of human D2 were identical, indicating that the second Sec and the following seven amino acids are not critical for its function (359).

Studies performed in rat cerebral cortex using cell fractionation and deiodinase activity measurements yielded that D2 was associated with membrane fractions (365). The availability of the D2 cDNA allowed more specific studies of the intracellular distribution of transiently expressed, catalytically active human D2 labeled with a FLAG epitope. The transiently expressed D2 is an integral ER membrane protein, and protease protection assays suggest that the N terminus remains in the ER lumen, whereas the C-terminal portion is in the cytosol (320). Immunofluorescent confocal microscopy of FLAG-D2-transfected HEK293 or neuroblastoma cells shows that transiently expressed human D2 colocalizes with glucose-regulated protein 78 (GRP78)/BiP, an ER resident protein, whereas FLAG-D1 is localized in or near the plasma membrane. Endogenous D2 also colocalizes with GRP78/BiP in the before mentioned MSTO-211H cells (126). This indicates that intrinsic sorting signals determine the differential subcellular localization of D2 and D1. Although the studies of D1 subcellular localization were performed with transiently expressed protein and need to be confirmed for native enzyme, these different subcellular localizations between D2 and D1 can explain the ready access of T3 generated from T4 by D2, but not D1, to the nuclear receptor, a phenomenon noted in the earliest studies of these two enzymes (345, 366, 367). However, studies in brain showed another important mechanism in the generation of nuclear T3. It is well known, that more than 50% of brain T3 is derived from local deiodination of T4 (368, 369), and in the rat brain as much as 80% of nuclear bound T3 is formed locally from T4 (120). Whereas the tanycytes and the astrocytes express D2 activity, D3 mRNA and T3 receptors are expressed in the neurons (370, 371). Nuclear T3 in neurons is derived in a paracrine fashion from D2 activity in tanycytes and astrocytes, whereas D3 activity in neurons protects these cells from exposure to excessive levels of T3. Thus, local T3 production in brain does not mean T3 production in the same cell as where T3 acts. This challenges the view that the differences in subcellular localizations between D2 and D1 account for the ready access of T3 production by D2, but not D1, to the nucleus. The recently identified MCT8 transporter is thought to be involved in uptake of astrocyte-derived T3 into neurons. This concept is supported by observations that mutations in this transporter result in the clinical phenotype of severe psychomotor retardation with high serum T3 levels (102). In these

patients the T3 transporter is deficient, i.e. there can be no T3 action in the neurons, whereas T3 breakdown is hampered as well, because T3 cannot enter the cell and therefore is not available for D3 action.

Intracellular regulatory pathways can be modified by selective proteolysis of key rate-limiting enzymes. This process is frequently mediated by the proteasome system in which different metabolic signals stimulate ubiquitin (Ub) conjugation of target proteins with subsequent selective uptake and proteolysis in proteasomes (372, 373). D2 is a key protein in a homeostatic system that controls the intracellular T3 concentration. It has been shown that D2 has a very short activity half-life (<1 h) that is further accelerated in cells exposed to its substrates T4 and rT3 and even high concentrations of T3. Site-directed mutagenesis showed the importance of enzyme-substrate interaction to occur to induce D2 proteolysis. Acceleration of protein degradation is lost in the D2 mutant Sec133Ala, which is not catalytically active, and decreased in the mutant Sec133Cys, which substrate affinity is greatly diminished (351, 374, 375). Since the loss of D2 activity is blocked by MG132, a proteasome uptake blocker, this indicates that substrate-induced changes in D2 molecule accelerate its processing by the proteasome (376). Direct evidence of D2 ubiquination was obtained in ts20 cells, a Chinese hamster ovary cell line containing a temperature sensitive ubiquitine-activating enzyme, where it was shown that D1 was not ubiquinated in agreement with the long D1 half-life (>12 h) (377). Furthermore, ubiquination and proteasomal degradation of D2 are likely to originate at the C terminus, which is exposed to the cytosol.

A missense SNP within the coding region of the D2 gene was recently described, featuring either threonine (Thr) or Ala at amino acid 92 (344, 378). A correlation with serum TSH was not observed (343). The data strongly suggested an association of the Thr92Ala with glucose intolerance and diabetes, suggesting a role of local T3 production by D2 in the regulation of energy metabolism. In the context of the so-called syndrome X, the complex of obesity, hypertension, insulin resistance and glucose intolerance/diabetes, this polymorphism may be of importance (344, 378).

Type III deiodinase (D3)

D3 is the third enzyme involved in reductive deiodination of thyroid hormones. In fact, it is the major T4 and T3 inactivating enzyme by catalyzing the conversion of T4 to rT3 and T3 to T2 by IRD (108, 114, 135, 162, 379). It was identified in the monkey hepatocarcinoma cell line (NCLP6E), and the first extensive physiological studies were performed in the rat central nervous system (CNS) (271, 272, 380, 381). In rodents and humans, D3 activity was

found in brain, skin, placenta, pregnant uterus, and in a variety of fetal structures, including the cerebrum, cerebellum, skin, liver, kidney and intestine (128, 130, 166, 167, 271, 274, 370, 382-386), although the highest activity found to date is in human infantile hemangiomas causing severe hypothyroidism (131). Also in various other species, such as chicken and fish, D3 activity is present in brain, liver and skin.

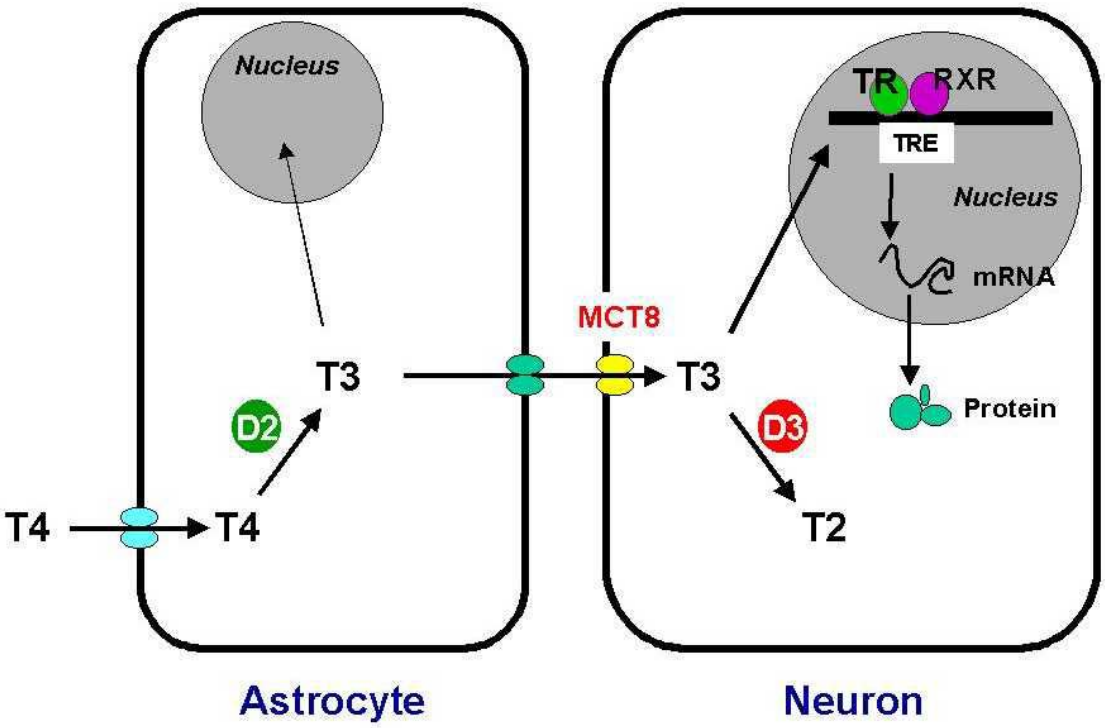

Fig. 8. Generation of neuronal nuclear T3 occurs in a paracrine fashion

After the cloning of D3 in X. laevis the corresponding cDNAs of many species (rat, human, chicken, tilapia) were isolated. The human D3 mRNA is 2066 nt and contains 220 bp of 5'-UTR, one open reading frame of 834 bp, and a 3'-UTR of 1012 bp (282). The deduced amino acid sequence predicts a protein of 278 residues, with a molecular mass of 31.5 kDa. Hydropathy analysis reveals a hydrophobic N-terminal portion consistent with a transmembrane domain. All D3 cDNAs cloned to date include a Sec-encoding TGA codon, as well as a SECIS element in the 3'-UTR. The conservation of D3 from X. laevis to humans implies that its role in regulating thyroid hormone inactivation is essential. Although the 2.3-kb band is the major mRNA in most tissues, at least four differently sized mRNAs from the rat CNS hybridize with the D3 cDNA, and dramatic changes in the relative intensity of these occur depending on thyroid status (370). It is not known whether the differences in transcript

sizes are due to the use of different poly (A) adenylation signals or degrees of polyadenylation.

In general, the expression of D3 is higher in embryonic and fetal life as compared to adult life, since during embryogenesis, D3 is critical for thyroid hormone homeostasis. Excess or premature exposure of the embryo to adult thyroid hormone levels can be detrimental and can result in malformations, altered growth, mental retardation and even death. Recently it was shown that D3 is highly expressed in the human syncytio- and cytotrophoblasts, endothelium of the placental and umbilical cord vessels, uterine decidua, i.e. the endothelium of the uteroplacental unit, and in the epithelium of the fetal tracheobronchial tree, small intestine, urothelium, and skin and, surprisingly, also in the nonpregnant human endometrium (169). The presence of D3 at maternal-fetal interfaces is consistent with its role in modulating the thyroid status of the human fetus and its expression in endometrium suggests that local regulation of thyroid status is important in implantation. Also, these results suggest that the local modulation of thyroid status is important at all stages of human development. Induction of D3 activity seems to begin immediately after implantation and is stimulated by 17 beta-estradiol and progesterone (387). Thyroid hormone is a major physiological regulator of brain development. The overall pattern of rat brain D3 distribution strongly suggests that D3 is primarily expressed in neurons but it is also present in primary astroglial cultures.

To evaluate the contribution of Sec144 to the catalytic properties of D3 enzyme, site-directed mutagenesis studies were performed, in which Sec144 (D3wt) was replaced either by Cys (D3Cys) or Ala (D3Ala) (Table 4C). This yielded a D3Ala protein that was enzymatically inactive and a D3Cys protein with reduced catalytic efficiency and altered substrate preference. This means, that the Sec residue in the catalytic center of D3 is essential for efficient IRD of T3 and T4 at physiological substrate concentrations (388).As with D2, substitution of Ser for Pro two positions downstream of the catalytic Sec residue turns D3 into a PTU-sensitive enzyme (335).

Table 4C. Structure-function relationship in D3 as studied by site-directed mutagenesis

Position	Residues	Effect	Ref
144	Sec>Ala	No activity	(388)
144	Sec>Cys	↑ Km T3 (5-fold), ↑ Km T4 (100-fold)	(388)
146	Pro>Ser	↑ Km T3, PTU sensitive	(335)
174	Glu>Ala	No activity	(335)

The numbering of amino acid residues corresponds to the position in human D3 [gi: 21903442]

The mouse Dio3 gene and its human homologue DIO3 code for D3. Both genes map to chromosomal regions which are known to include imprinted genes (chromosome 12F1 and 14q32, respectively) (389-391). By studying Dio3 knock-out mice, it has recently been shown, that the Dio3 gene is subject to genomic imprinting and is preferentially expressed from the paternal allele in the mouse fetus. Also a non-coding gene that overlaps the Dio3 gene and is transcribed antisense from Dio3 was described (392). Such non-coding antisense transcripts are often associated with imprinted genes, and may be involved in the imprinting process. The Dio3 gene belongs to a large cluster of imprinted genes detected on mouse chromosome 12 and human chromosome 14. Alterations in genomic imprinting of chromosomes 12 and 14 in mice and humans, respectively, lead to abnormal phenotypes (391, 393, 394). To what extent alterations in D3 expression contribute to these abnormal phenotypes remains to be investigated.

Alternative splicing

The sequencing of the human genome has raised important questions about the nature of genomic complexity. It was widely anticipated that the human genome would contain a much larger number of genes than that of Drosophila. The report of only 32,000 human genes came as a surprise. This basic disparity indicated that the number of human expressed sequence (mRNA) forms was much higher than the number of genes, suggesting a major role for alternative splicing as a mechanism for combinatorial gene output. Alternative splicing seems to occur very frequently (395, 396), which indicates that alternative splicing is far more functionally important than previously thought. Recent studies indicate that 70-88% of alternative splices change the protein product. This can lead to critical developmental decisions such as sex and death, since many alternatively spliced products are themselves important regulators (396). Furthermore, programmed cell death (apoptosis) is influenced by alternative splicing events whereas alternative splicing may be most important in complex systems where information must be processed differently at different times (such as immune tolerance, or development) or a very high level of diversity is required (such as axonal guidance) (395). Therefore, alternative splicing can be involved in a number of patho-physiological mechanisms.

AIM AND OUTLINE OF THE THESIS

In the first part of this thesis we describe our study of structure-function relationship of deiodinases. This can be done either by comparison of sequence information and enzyme activities of deiodinases from different species or by site-directed mutagenesis. Both approaches were followed. These studies were performed in the pig and the cat. Furthermore, thyroid state-dependent regulation of deiodinase expression was studied in the pig. Pigs were rendered thyrotoxinaemic by oral administration of T4, whereas they were made hypothyroid by treatment with methimazole. In Chapter 2 the results on cat D1 are presented. Cat D1 was studied because of the report that this enzyme has a very low activity towards rT3, which is usually the preferred substrate for D1(397). Besides cloning of cat D1 site directed mutagenesis is performed. Chapter 3 summarizes our findings on characteristics of iodothyronine deiodinases in the pig. Porcine deiodinases are cloned and the sequences are compared with deiodinases from other species. Moreover, thyroid-state dependent regulation of the porcine deiodinases is studied.

Since the number of human expressed mRNA forms is much higher than the number of genes there is good evidence that alternative splicing plays a very significant role in the complexity of genomic function (395, 398). Until now alternative splicing has only been described for human D2. This comprised alternative splicing in the 5'-UTR part with alternative transcription start sites (399), but also insertion of an additional sequence in the intron of human D2 yielding 2 novel exons between exon 1 and 2 of the human D2 gene (400). However, until now there has not been any report about alternative splicing in D1. In this part of the thesis the possibility of alternative splicing in human D1 is explored. Chapter 4 summarizes our findings on alternative splicing in hD1 in adult and fetal human liver as well as adult human thyroid and kidney. Furthermore, in another approach to identify possible splice variants we screened Genbank to search for hD1 variants.

The second part of the thesis is about the role of thyroid hormone metabolism in the pathogenesis of heart failure. The aim of this part of the study is to investigate the role of local thyroid hormone metabolism in the development of compensated hypertrophy and heart failure. Cardiac performance is dependent on adequate intracardial levels of T3. Regulation of intracardial T3 levels depends on serum T4 and T3 levels, cellular uptake of T4 and T3, intracellular conversion of T4 to T3 and inactivation of these compounds. As presented in the general introduction section D1 activity in cardiomyocytes of rodents has been identified, whereas D3 activity was detected in human fetal heart samples. Again, in rodents recently D2

activity regulated by thyroid status was described, whereas, despite the identification of D2 mRNA, till now no D2 activity could be identified in human cardiomyocytes. Finally, T3 action in the cardiomyocyte can be inhibited by a change in receptor expression. In heart failure, Ca-handling is disturbed and T3-regulated genes show a hypothyroid regulation pattern, indicating local hypothyroidism. Our hypothesis is, that altered conversion or degradation of thyroid hormone in the cardiomyocyte itself may be an important mechanism resulting in reduced local biological activity of thyroid hormone in pathological hypertrophy and heart failure.

For this study we used an animal model of monocrotaline (MCT) induced pulmonary hypertension, in which both compensatory hypertrophy and heart failure can develop. MCT is a pyrrolizidine alkaloid, a phytotoxin derived from *Crotolaria spectabilis*. MCT has to be activated by the liver to monocrotaline pyrrole (MCTP) (401). Whereas in aqueous solution the half-life of this intermediate is only seconds, binding to red blood cells allows stabilization and transport to the lungs. In the lungs, MCTP induces proliferative pulmonary vasculitis (402), thereby increasing vessel wall thickness (403), resulting in an increased pulmonary resistance. This results in right ventricular pressure overload. The MCT model is a widely used animal model for pulmonary hypertension (402, 404-406) or right ventricular cardiac hypertrophy (407). Cardiac gene expression, contractile function, Ca-homeostasis, plasma and local thyroid hormone levels and ventricular deiodinase activity were measured in this model.

In Chapter 5 pressure overload is induced by subcutaneous injection of 40 mg/kg MCT and analysis takes place after four weeks. MCT administration yields a group of animals developing right ventricular hypertrophy (HYP) without signs of heart failure and a group developing severe congestive heart failure (CHF). A control population is formed by administration of a single subcutaneous saline injection. Chapter 5 summarizes our findings on deiodinase activities in the three experimental groups in the left versus the right ventricle. Furthermore, cardiac parameters, i.e. expression levels of the Ca^{2+}-pump of the sarcoplasmic reticulum, Ca^{2+}-ATPase type 2a (SERCA2a), and the contractile proteins, myosin heavy chain α (MHCα) and myosin heavy chain β (MHCβ), are measured as well as plasma thyroid hormone parameters.

In Chapter 6 a two-dose protocol is used to induce HYP and severe CHF (30 mg versus 80 mg of MCT, respectively). This allows analysis of changes in gene expression, functional properties and deiodinase activities at earlier time points, i.e. at two, three and four weeks in animals before they develop obvious signs of CHF. Furthermore, the effect on tissue T3 content is studied as well.

In the General discussion (Chapter 7) the results of the studies reported in this thesis are evaluated and an outlook to future research is presented.

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CHAPTER 2

MOLECULAR BASIS FOR THE SUBSTRATE SELECTIVITY OF CAT TYPE I IODOTHYRONINE DEIODINASE

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The cat D1 iodothyronine deiodinase sequence has been submitted to the GenBank database under accession number AY347714

ABSTRACT

The type I iodothyronine deiodinase (D1) catalyses the activation of thyroxine (T4) to 3,5,3'-triiodothyronine (T3) as well as the degradation of 3,3',5'-triiodothyronine (rT3) and sulfated iodothyronines. A comparison of the catalytic activities of D1 in liver microsomal preparations from several species revealed a remarkable difference between cat D1 on one hand and rat/human D1 on the other hand. The K_m of cat D1 for rT3 (11 μM) is 30-fold higher than that of rat and human D1 (0.2 – 0.5 μM). Deiodination of rT3 by cat D1 is facilitated by sulfation (V_{max}/K_m rT3 = 3 and V_{max}/K_m rT3S = 81). To understand the molecular basis for the difference in substrate interaction the cat D1 cDNA was cloned, and the deduced amino acid sequence was compared with rat/human D1 protein. In the region between amino acid residues 40 and 70 of cat D1 various differences with rat/human D1 are concentrated. By site-directed mutagenesis of cat D1 it was found that a combination of mutations was necessary in order to improve the deiodination of rT3 by cat D1 enzyme. For efficient rT3 deiodination a Phe at position 65 and the insertion of the Thr-Gly-Met-Thr-Arg (48-52) sequence as well as the amino acids Gly and Glu at position 45-46 are essential. Either of these changes alone resulted in only a limited improvement of rT3 deiodination. At the same time the combination of the described mutations did not affect the already quite

efficient outer ring deiodination of rT3S or the inner ring deiodination of T3S, while each of the described changes alone did affect rT3S deiodination. Our findings suggest great flexibility of the active site in D1, which adapts to its various substrates. The active site of wild type cat D1 is less flexible than the active site of rat/human D1 and favors sulfated iodothyronines.

INTRODUCTION

The major route for the production of the active form of thyroid hormone, T3, is by enzymatic outer ring deiodination (ORD) of the prohormone, T4 in peripheral tissues. Alternatively, inner ring deiodination (IRD) of T4 produces the inactive metabolite reverse T3 (rT3). Three membrane-bound iodothyronine deiodinases have been identified (1-3). The type I iodothyronine deiodinase (D1) selenoprotein is expressed in liver, kidney, thyroid and pituitary. This enzyme is responsible for a large part of the peripheral production of T3 from T4 in euthyroid animals (4-5). Remarkably, D1 is capable of both ORD and IRD of T4, and shows preference for rT3 as the substrate (6-7). D1 activity *in vitro* is stimulated by thiol compounds such as dithiotreitol (DTT) and is inhibited by propylthiouracil (8). N-bromoacetyl-[^{125}I]-T3 has proven to be a useful affinity label of D1, allowing the specific labeling of the 27 kDa protein in microsomal fractions (9-10). Molecular sieve chromatography and sedimentation analysis of the detergent solubilized D1 yielded a ~ 50 kDa active enzyme preparation, suggesting that the D1 protein is composed of a homodimer of 27 kDa subunits (11-12). The functional significance of dimerization is unknown, since it has been shown that dimers containing only one wild-type partner are catalytically active (12-13). There is general agreement that D1 is an integral membrane protein, but different cellular localizations have been found. In kidney cells D1 is present in the plasma membrane (12, 14-15), while in liver cells D1 is present in the endoplasmic reticulum with its active site oriented to the cytoplasm (16-17). In transiently transfected HEK-293 cells D1 was localized in the plasma membrane as determined by immunofluorescence confocal microscopy (18).

More detailed structure-function analysis became possible after cloning of the D1 cDNA (6). The D1 protein contains a single selenocysteine residue (SeC) in the catalytic center, which is essential for efficient catalysis (6, 19-20). A comparison of the deduced amino acid sequences of rat (6), human (21), dog (22), chicken (23), tilapia (24) and killifish (25) D1 reveals that only a single domain in the N-terminus is sufficiently hydrophobic to qualify as a transmembrane sequence. *In vitro* translation studies using pancreatic microsomes showed that the transmembrane domain of rat D1 is located between basic amino acids at positions 11 and 12 and a group of charged residues at positions 34-39 (26). The presence of essential active site histidine (His) residue(s) was postulated on the basis of experiments with histidine-directed reagents (27). Systematic site-directed mutagenesis studies of the four histidine residues in rat D1 showed that mutagenesis of His174 caused a significant increase in the K_m for rT3 deiodination, compatible with the formation of an imidazolium-selenolate ion pair

(28). Comparative functional-structural analysis of human and dog D1 enzymes showed that amino acids between residues 30 and 70 of dog D1 account for the difference in K_m value for rT3 ORD between dog and human D1 (22). Dog D1 has an approximately 30-fold higher K_m for rT3 ORD than human D1 (22, 29). More detailed studies demonstrated that it is mainly the Phe65Leu substitution, which explains the slow ORD of rT3 by dog D1 vs. human and rat D1 (22, 30).

Investigations on iodothyronine deiodination by cat liver and kidney microsomal fractions revealed that the ORD of rT3 is even slower than that by dog liver microsomes (31). The K_m for ORD of rT3 by cat D1 was at least 500-fold higher than that of rat D1 (> 100 μM vs. 0.2 μM), while cat and rat D1 deiodinated T4 at a similar rate with equal K_m values (2 μm). In kittens reared on low-selenium diet plasma total T4 increased while total T3 decreased (32), suggesting that cat D1 is a selenoprotein just as rat and human D1 albeit with differential substrate selectivity.

The present studies were undertaken to obtain detailed information about the substrate-binding site in D1 protein. We have therefore isolated a D1 cDNA from cat liver and expressed this enzyme in COS cells in order to analyze its kinetic properties with different iodothyronine substrates. By comparing the cat and rat/human D1 primary sequences and subsequent site-directed mutagenesis experiments with cat D1 we have identified a region, which is involved in iodothyronine substrate interaction.

MATERIALS AND METHODS

Materials

Nonradioactive iodothyronines were obtained from Henning (Berlin, Germany). [3'-^{125}I]T3 (2000 mCi/μmol) and [3',5'-^{125}I]T4 (1200 mCi/μmol were obtained from Amersham Pharmacia Biotech (Little Chalfont, UK). [3',5'-^{125}I]reverseT3 (rT3) and [3'-^{125}I]T2 were prepared by radioiodination of 3,3'-T2 using the chloramine-T method as described (33). Radioactive as well as nonradioactive rT3S, T3S and T2S were prepared by reaction of rT3, T3 or T2 with chlorosulfonic acid as described (34). Radioactively labeled *N*-bromoacetyl-T3 (BrAc[^{125}I]T3) was synthesized from bromoacetylchloride and [3'-^{125}I]T3 as described (35). *Pfu* (*Pyrococcus furiosus*) thermostable DNA-polymerase, *DpnI* restriction endonuclease and pGEM-T vector were obtained from Promega Corporation (Madison, WI). XL-10 ultracompetent *Escherichia Coli* cells were obtained from Stratagene (La Jolla, CA). Synthetic oligonucleotides, recombinant *Taq* DNA-polymerase, M-MLV reverse transcriptase and cell culture media were ordered from Invitrogen-Life Technologies (Paisley, UK).

Human and animal liver tissue

Normal adult human liver tissue was obtained at surgery for liver tumors. Approval was obtained from the Medical Ethical Committee of the Erasmus Medical Center. All animal protocols were reviewed and approved by the institutional animal care and use committees of the School of Veterinary Medicine, Utrecht University (cats, dogs) or the Erasmus Medical Center (pigs, rats).

Assay of ORD and IRD activity in liver microsomes

Liver tissue from different species (human, pig, rat, cat and dog) was homogenized and microsomal fractions were prepared by differential centrifugation as described (36). Microsomal pellets were dissolved in 100 mM phosphate (pH 7.2), 2 mM EDTA buffer containing 1 mM DTT (PED1). Protein concentrations (15 – 25 mg/ml) were determined with the Bradford method using the Bio-Rad protein assay reagent and BSA as standard. Aliquots of microsomes were snap-frozen on dry ice, and stored at – 80 C.

The ORD activity was measured by incubation of diluted microsomal fractions (final concentration 10 – 50 μg protein /ml) in P100E2D10 buffer (100 mM phosphate, pH 7.2; 2 mM EDTA, 10 mM DTT) with 10 nM (100000 cpm) ^{125}I-labeled substrate (rT3, rT3S, T2S)

followed by isolation and quantitation of the $^{125}I^-$ released as described (30, 37). The ORD of rT3 was analyzed in more detail. Microsomal fractions were incubated for 60 min at 37 C with 0.01 – 30 μM ^{125}I-rT3 (100000 cpm) in P100E2D10. Values for V_{max} and K_m were estimated using double reciprocal plots as previously described (37).

The IRD assay with T3S is based on the determination of product formation (^{125}I-labeled T2S and $^{125}I^-$) by reverse-phase HPLC analysis of reaction mixtures containing outer-ring labeled ^{125}I-T3S. Diluted microsomal fractions (50 μg protein/ml) were incubated with 10 nM (200000 cpm) ^{125}I-labeled T3S for 60 min at 37 C in P100E2D10. The reactions were stopped by addition of methanol (1:1), and analyzed by HPLC as described (38).

RT-PCR cloning of cat D1 cDNA and construction of expression vector

Total RNA was isolated from cat liver tissue with TRIzol reagent (Gibco), and cDNA was obtained using random hexamer primers and M-MLV reverse transcriptase. The coding sequence of cat D1 was cloned by PCR with oligonucleotide primers derived from the rat/human D1 cDNA sequences around the translational start codon (5'-ATGGGGCTGTCCCAGCTA), and the stop codon (5'-TTAACTGTGGAGCTTTTC). The PCR products obtained were subcloned in the pGEM-T vector and sequenced in both directions.

Because a SECIS element (selenocysteine insertion sequence element) is required for incorporation of SeC in selenoproteins, we prepared chimeric constructs in which the cat D1 cDNA was inserted 5' to the SECIS element of the rat D1 gene. For this purpose, the G21-pcDNA3 rat D1 expression vector (6) was digested with *Hind*III, and the 6 kb DNA band containing vector DNA plus 0.7 kb of the rat D1 3'-UTR (including the SECIS-element) was isolated from a preparative agarose gel. The D1 sequence of the pGEM plasmid was amplified with primers containing flanking *Hind*III restriction sites (italics): 5'-C*AAGCTT*<u>GCCACCATGG</u>GGCTGTCCCAGCTA (Kozak start consensus underlined) and 5'-C*AAGCTT*<u>TTA</u>ACTGTGGAGCTTTTC (stop codon underlined) and cloned in pGEM-T vector. The pGEM vector containing cat D1 cDNA was digested with *Hind*III, and the isolated fragment was cloned into the prepared rat D1-SECIS-pcDNA3 vector.

Site-directed mutagenesis of cat D1

The cat D1 expression vector was used as template for site-directed mutagenesis via the circular mutagenesis procedure, followed by selection for mutants by *Dpn I* digestion (39-40). The desired mutations were introduced successively. In the first round of mutagenesis the

L60Y61 wt sequence was changed via overlapping sense and antisense primers containing the nucleotide changes needed to produce the following mutants:
F60Y61 (sense 5'CAACTGGGCCCCAACTTTTACAGCGTGCAGTATTTCTGG)
Y60Y61 (sense 5'CAACTGGGCCCCAACTTACTACAGCGTGCAGTATTTCTGG)
F60F61 (sense 5'CAACTGGGCCCCAACTTTTTTCAGCGTGCAGTATTTCTGG)
L60L61 (sense 5'CAACTGGGCCCCAACTCTGTTGAGCGTGCAGTATTTCTGG)
Circular mutagenesis reactions were performed with 50 ng plasmid template and 2 U *Pfu* DNA polymerase. The cycling protocol consisted of 30 sec 95 C, 1 min 55 C, 14 min 68 C for 18 cycles using a Perkin Elmer model 480 PCR machine. The products were incubated with 10 U *Dpn I* for 2 h at 37 C, and transformed to competent *E.Coli* XL-10 cells according to manufacturers instructions. Plasmid DNA isolated from several clones was sequenced to verify that the desired mutation had been generated, and that no unwanted mutations were introduced. Plasmids were maintaned in *E. Coli* DH5α cells and purified for transfection with QIAfilter cartridges (Qiagen, Hilden, Germany).

In the second round of mutagenesis the TGMTR insertion between amino acid residues 47 and 48 was introduced in wt cat D1 and the described mutants using the sense oligo-nucleotide primer 5'GCCATGAACCGGAAGACCGGAATGACCAGGAACCCCCACTTTTCC (insertion underlined).

In the third round of mutagenesis the N45R46 to G45E46 mutation was introduced in wt cat D1 (L60Y61) and the F60Y61, F60F61, L60Y61 + TGMTR insertion, F60Y61 + TGMTR insertion and the F60F61 + TGMTR insertion D1 mutants using the sense oligonucleotide primer 5'CACATCGTGGCCATGGGCGAGAAGAACCCCCACTTTTCC (mutants without TGMTR insertion) or 5'CACATCGTGGCCATGGGCGAGAAGACCGGAATGACCAGG (mutants with TGMTR insertion).

Expression of D1 protein

The wt and mutant D1 enzymes were expressed in COS cells (65 cm^2 dishes) after DEAE-dextran-mediated transfection (8 μg/dish) of the expression vectors (37). COS cells were grown in DMEM-Ham's F-12 medium containing 10% FBS and 40 nM sodium selenite. Two days after transfection the cells were rinsed with PBS and collected in 0.25 ml P100E2D10 buffer, sonicated, aliquoted and stored at – 80 C.

Assay of ORD activity in COS cell homogenates

Two different ORD assays were done, involving 1) incubation with [3',5'-^{125}I]rT3 and isolation and quantitation of the $^{125}I^-$ released or 2) incubation with [3',5'-^{125}I]rT3S and isolation and quantitation of the $^{125}I^-$ released with subsequent correction via HPLC analysis of reaction products for $^{125}I^-$ released from [3'-^{125}I]T2S.

1) Varying amounts of homogenates (50 – 250 μg protein/ml) were incubated for 60 min at 37 C with 0.1 – 30 μM rT3 (100000 cpm) in 0.1 ml P100E2D10 buffer. Reactions were stopped by addition of 0.1 ml 5% BSA on ice. Protein-bound iodothyronines were precipitated by 10 % TCA on ice, and the radioiodide in the supernatant was isolated by chromatography on Sephadex LH-20 mini columns as described (30). Protein was adjusted to consume less than 30% of substrate, and deiodination was corrected for nonenzymatic deiodination in blank incubations with homogenates of mock transfected COS cells. The radioiodide production was multiplied by two to account for the random labeling and deiodination at the 3' and 5' positions of the substrate.

2) Varying amounts of homogenate (20 – 100 μg protein/ml) were incubated for 60 min at 37 C with 30 – 2000 nM ^{125}I-rT3S (50000 cpm) in 0.1 ml P100E2D10. The amount of $^{125}I^-$ released was determined in the same way as described for rT3 ORD.

In parallel incubations 100 – 300 nM ^{125}I-rT3S (200000 cpm) was incubated in the same manner and the reaction was stopped by the addition of 0.1 ml methanol. The supernatant was mixed (1:1) with 0.02 M ammonium acetate (pH 4.0) and applied to a Symmetry C18 column connected to a Alliance HPLC system (Waters, Milford, MA) which was eluted with a 20 min linear gradient of 24 – 29 % acetonitrile followed by a 6 min gradient of 29 – 50% acetonitrile in 0.02 M ammonium acetate. Radioactivity in the eluate was monitored online using a Radiomatic A-500 flow scintillation detector (Packard, Meriden, CT). The amount of $^{125}I^-$ released as determined by chromatography over LH-20 columns was multiplied with the correction factor ($^{125}I^-$ cpm + ^{125}I-T2S cpm) / ($^{125}I^-$ cpm) calculated from the HPLC analysis. The correction factor was 1.5 – 1.7 in most cases. In this way only the conversion of rT3S to T2S is taken into consideration.

Assay of IRD activity in COS cell homogenates

IRD activity was measured with outer ring ^{125}I-labeled T3S. In this assay IRD activity is the sum of ^{125}I-T2S as well as $^{125}I^-$ formed. The latter is formed by outer ring deiodination of ^{125}I-T2S. Homogenates (0.14 – 0.17 mg protein/ml final concentration) were incubated for 60

min at 37 C with 0.1 μM ^{125}I-T3S (200000 cpm) in 0.1 ml P100E2D10. The reaction was stopped by the addition of methanol (1:1) on ice, and the reaction mixtures were analyzed by reverse-phase HPLC as described above for rT3S.

Measurement of serum iodothyronines

Total T4, T3 and rT3 were analyzed by radioimmunoassay as previously described (33).

Polyclonal antiserum production and Western blotting

Polyclonal antisera were raised in rabbits by Eurogentec SA (Seraing, Belgium) against the KLH conjugate of the synthetic peptide (C)NPEEVRAVLEKLHS (human D1 amino acid residue 236-249). Antiserum (designated 1068) from the final bleed was used without further purification. Homogenates from transfected COS cells (20 – 40 μg protein) were separated on 12% SDS-PAGE gels in the Mini-Protean III cel (Bio-Rad Laboratories, Hercules, CA) according to manufacturer's instructions. After electrophoresis the proteins were blotted to nitrocellulose membranes and probed with antiserum 1068 (1:500) as described previously (38). The intensity of the D1 protein bands was analyzed by densitometry. In control experiments it was shown that the antiserum does not detect human D2 or D3 protein.

Affinity labeling of D1 with N-bromoacetyl-[^{125}I]T3

BrAc[^{125}I]T3 (1500 mCi/μmol) was synthesized as described (35), and HPLC analysis demonstrated that the purity was at least 85% with unreacted [^{125}I]T3 as the main contaminant. Solutions of BrAc[^{125}I]T3 (100000 cpm, 0.03 pmol) in ethanol were pipetted into microcentrifuge tubes, and the solvent was evaporated by a stream of nitrogen. After addition of 25 μl P100E2D10 and vortexing, the COS cell homogenates (50 μg protein) were added in a total volume of 50 μl P100E2D10. The mixtures were incubated for 20 min at 37 C. Reactions were terminated by addition of SDS-PAGE gel-loading buffer, and samples were analyzed by SDS-PAGE (12% gel) followed by autoradiography to Kodak BioMax MS film at – 80C with intensifying screen. After autoradiography, lanes were excised from the gel and the slices were counted for radioactivity. The radioactivity in slices from lanes of non-transfected cells was subtracted. The net incorporation in cat D1 wt protein was 5 – 6 % of the added amount of ^{125}I-labeled BrAcT3.

RESULTS

Enzymatic activities of D1 from different species

Deiodination of various iodothyronine derivatives by human, porcine, rat, cat and dog liver microsomes was studied. The results obtained with various substrates (rT3, rT3S, T2S, T3S) at a concentration of 10 nM are presented in Figure 1. It is obvious that human, and especially rat and porcine D1 prefer rT3 as substrate while cat and dog D1 prefer sulfated rT3 (rT3S) as substrate. Furthermore, while dog D1 slowly deiodinates rT3 compared to rat, human and porcine D1, it is obvious that cat D1 does not deiodinate rT3 at all under the conditions used. The kinetics of the ORD of rT3 were studied in detail by incubation of liver microsomes with varying rT3 concentrations (Table 1). The K_m values for ORD of rT3 by cat D1 are 22 – 50 times higher compared to human, porcine and rat D1. Cat D1 is even less efficient than dog D1 with regard to ORD of rT3 as reflected in the 3-fold higher K_m value and the 5-fold lower V_{max} value.

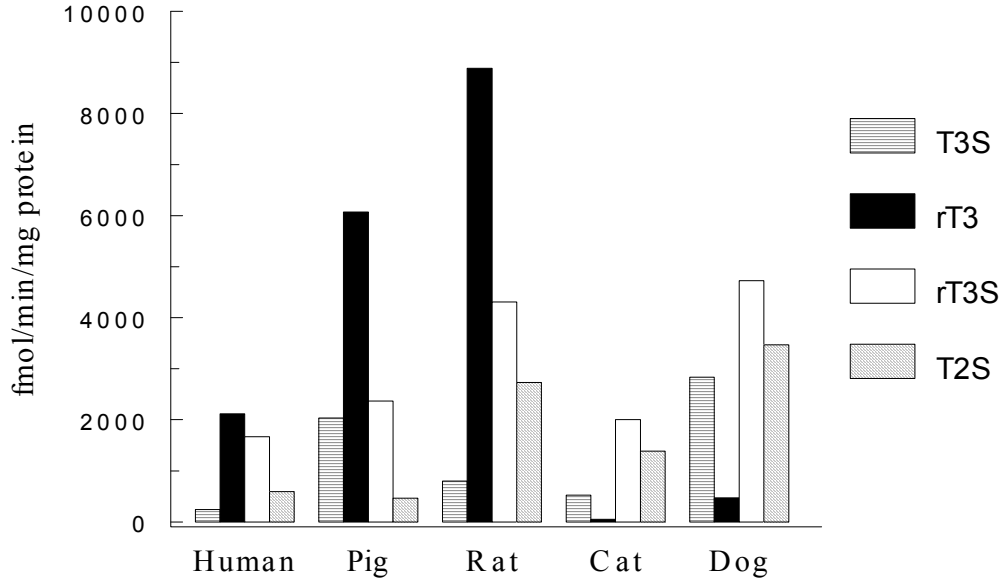

Fig 1. Rate of deiodination of different iodothyronine derivatives by human, pig, rat, cat and dog liver microsomes. ORD activity was measured by incubation of diluted microsomal fractions in P100E2D10 with 10 nM (100000 cpm) ^{125}I-labeled substrate (T3S, rT3, rT3S, T2S) for 60 min at 37 C. IRD activity was measured by incubation of diluted microsomal fractions in P100E2D10 with 10 nM (200000 cpm) ^{125}I-T3S for 60 min at 37 C. Analysis was performed by reverse-phase HPLC.

The slow deiodination of rT3 by cat liver microsomes could in part be the consequence of a low D1 protein expression level and/or it could be an intrinsic property of this enzyme. In order to answer this question Western blots were made of cat, porcine and human liver microsomal preparations using a newly developed D1 antiserum. This antiserum recognizes an epitope at the C-terminus of human D1 protein, which is conserved in porcine, and cat D1 proteins. The antiserum detects proteins with an apparent molecular mass of 24 - 25 kDa in cat liver microsomes and 27 - 28 kDa in human and porcine liver microsomes (Figure 2).

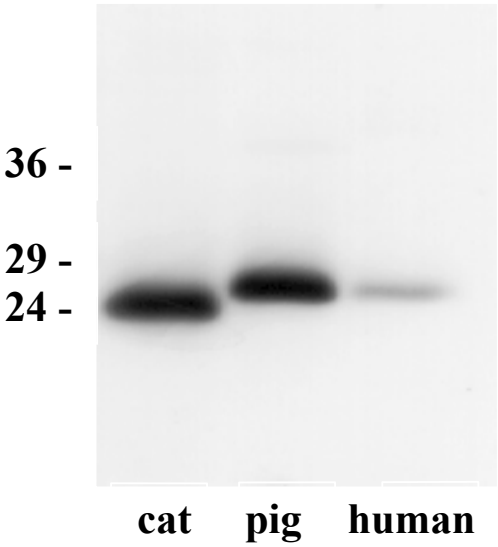

Fig. 2. Western blot analysis of cat, porcine and human microsomes. Twenty micrograms of microsomal protein were probed with a new anti-D1 antiserum generated in our laboratory against an 14-amino-acid peptide corresponding to the C-terminal sequence of human D1 (see Materials-Methods). The signal was absent when the microsomes were probed with preimmune serum (not shown). Migration distances of molecular mass markers (kilodaltons) are indicated

The molecular mass of human and porcine D1 is in line with the value of 27 – 28 kDa reported after affinity labeling with ^{125}I-BrAcT3 of human, porcine and rat liver microsomes (9). For cat and dog liver D1 somewhat lower molecular mass values of 25 – 26 kDa were found after affinity labeling (9, 31). The expression level of cat D1 protein is almost equal to that of porcine D1 as judged by Western blot (see Figure 2), nevertheless the rT3 ORD activity by cat D1 is much lower than that of porcine D1. In other words, the slow deiodination of rT3 by cat D1 is an intrinsic property of the enzyme, as was also reflected in the increased K_m value for rT3 deiodination (Table 1).

Table 1. Kinetic characteristics of liver D1 (rT3 ORD).

Species	K_m (rT3) μM	V_{max} (rT3) pmol/min.mg
human	0.50	120
porcine	0.16	100
rat	0.22	1520
cat	11	54
dog*	4.2	278

*Values for dog liver are taken from ref. 30

Serum rT3 concentration in euthyroid cats

The rather inefficient deiodination of rT3 under our *in vitro* conditions suggests that hepatic metabolism of rT3 by D1 cannot proceed at a significant rate in the cat. This could be reflected in elevated serum rT3 concentrations. In an effort to investigate this, serum T4, T3 and rT3 levels were measured in sera from two cats and compared to mean reference values for cats and humans (Table 2). While the T3/T4 ratios for cats and humans are in the same range, the rT3/T4 ratio's for cats are elevated compared to humans. Since only two cats were investigated these results should be interpreted with caution, but they suggest that rT3 metabolism in the cat is less efficient than in humans.

Table 2. Serum iodothyronine levels in cat and human

Sample	T4 nmol/L	T3 nmol/L	rT3 nmol/L	T3/T4 x 100	rT3/T4 x 100
cat 1	28	0.81	0.50	2.89	1.79
cat 2	16	0.63	0.33	3.94	2.06
cats[(1)]	24	0.70		2.92	
humans[(1)]	105	2.20	0.28	2.09	0.26
humans[(2)]	93	1.97	0.24	2.12	0.26

(1) Mean reference values from ref. 41
(2) Mean reference values Erasmus MC

Cloning and characterization of cat D1

Based on homology between conserved amino acid sequences in known D1 proteins (6, 21-25) oligonucleotide primers were designed corresponding to sequences around the start and stop codons (see Materials and Methods). RT-PCR with these primers on cat liver total RNA produced a DNA fragment of about 800 bp, which was subcloned and sequenced. Alignment of the deduced amino acid sequence with the reported sequences of other deiodinases (Figure 3) revealed 80% overall identity with human, rat and porcine D1. The same alignment showed 92% overall identity with dog D1. Deiodinases contain a single selenocysteine residue (SeC) in the catalytic center, which is essential for catalytic activity (19-20). The core catalytic center of about 15 amino acid residues around the SeC residue is completely conserved between rat, man, pig, dog and cat D1. This indicates that the observed differences in substrate preference are probably caused by amino acid variations in other functional domain(s).

Transient expression in COS cells of the cDNA fragment encoding cat D1 carried in the pcDNA3 expression vector as a chimeric construct with the rat D1 SECIS element, resulted in the synthesis of a functional deiodinase (Table 3). The K_m for ORD of rT3 by the recombinant cat D1 enzyme was as high as that of the native D1 enzyme (15 vs. 11 μM). The K_m of cat D1 is 80 times higher than rat D1, and assuming equal protein expression levels it appears that the catalytic efficiency (V_{max}/K_m ratio) of cat D1 is 70-fold reduced compared to rat D1. The ORD of rT3 by cat D1 is facilitated by sulfation (V_{max}/K_m rT3 = 3 and V_{max}/K_m rT3S = 81). Both native and recombinant cat D1 ORD activity is effectively inhibited by PTU, and the IC_{50} value is 5 – 10 μM (not shown).

Site-directed mutagenesis of cat D1 and ORD of rT3 or rT3S

Aim of the mutagenesis experiments was to improve the ORD of rT3 by cat D1 without affecting the efficient ORD of rT3S. In other words, to create a mutant cat D1 enzyme with characteristics similar to those of rat D1 which deiodinates rT3 and rT3S with equal efficiency (see Table 3). Between amino acid residues 40 and 70 several remarkable differences exist between cat and rat/human D1, in particular the deletion of the TGMTR sequence (48-52) in cat D1 (Figure 4). Other differences are the substitution of F65L / F66Y (human/rat vs. cat D1) and the substitution of G45N / Q46R (rat vs. cat D1) or G45N /E46R (human vs. cat D1).

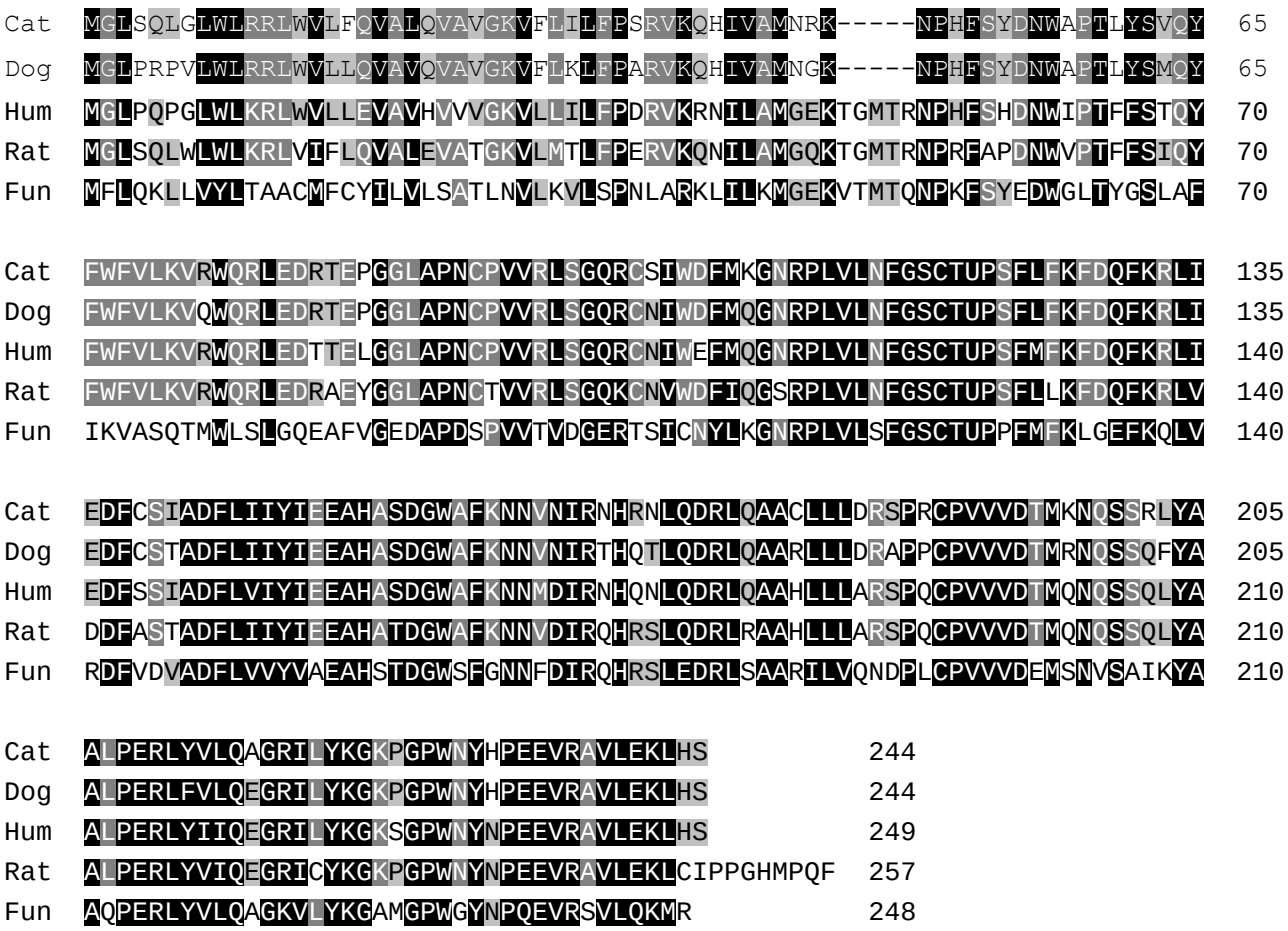

Fig. 3. Alignment of the deduced amino acid sequences of cat, dog, human, rat and *Fundulus heteroclitus* (killifish) type I iodothyronine deiodinase (D1). The selenocysteine residue in the catalytic center is indicated by U (residue 121 in cat D1).

Table 3. Kinetic characteristics of recombinant D1 enzymes (ORD)

Deiodinase	K_m (rT3)	V_{max} (rT3)	K_m (rT3S)	V_{max} (rT3S)
	μM	pmol/min.mg	μM	pmol/min.mg
cat	15	37	0.7	57
rat	0.19	38	0.19	42
human*	0.32	-	-	-

*Value taken from ref. 30

	40 45 48 52 65 70
Rat	NILAM**GQ**K**TGMTR**NPRFAPDNWVPT**FF**SIQY
Human	NILAM**GE**K**TGMTR**NPHFSHDNWIPT**FF**STQY
CWT	HIVAM**NR**K**-----**NPHFSYDNWAPT**LY**SVQY
CM1	FF
CM2	FY
CM3	YY
CM4	LL
CM5	TGMTR
CM6	TGMTR FF
CM7	TGMTR FY
CM8	TGMTR YY
CM9	TGMTR LL
CM10	GE
CM11	GE FF
CM12	GE FY
CM13	GE TGMTR
CM14	GE TGMTR FF
CM15	GE TGMTR FY

Fig. 4. Amino acid sequence residue 40 – 70 of cat D1 constructs (only changes compared to cat D1 wt are indicated). CWT = cat D1 wt, CM = cat mutant

Surprisingly, the TGMTR insertion (CM5) in itself does not improve deiodination of rT3. Only in combination with the L65F substitution further improvements in catalytic efficiency are obtained (CM6 and CM7). Similarly, the combined N45G and R46E substitutions provide only a small improvement in deiodinase activity (CM10), but in combination with the L65F or TGMTR insertion greater increases in catalytic efficiency are obtained (CM11, CM12 and CM13). Combination of all three mutations that is the L65F, N45G and R46E substitutions plus the TGMTR insertion strongly improved the catalytic efficiency (CM14 and CM15). These mutations seem to influence each other in a positive fashion. For example, the TGMTR insertion alone has no effect (CM5) but the TGMTR insertion in CM11 (giving CM14) and CM12 (giving CM15) results in a 5-fold increase in catalytic efficiency. The K_m for ORD of rT3 by CM14 and CM15 is ≤ 1 μM, i.e. a >15-fold decrease vs. wt (Table 4). A Phe residue at position 65, as in rat/human D1, is important for efficient ORD since CM13 is clearly less active than CM15. In the absence of Phe at position 65, as in CWT, a Tyr at position 66 is essential for activity since CM4 and CM9 are inactive.

Table 4. Kinetic characteristics of mutant D1 enzymes with rT3 and rT3S.

Enzyme	K_m rT3 μM	V_{max} rT3 pmol/min.mg	V_{max}/K_m[1]	K_m rT3S μM	V_{max} rT3S pmol/min.mg	V_{max}/K_m[1]
CWT	15	37	3 / 3	0.7	57	81 / 81
CM1	14	78	6 / 7	0.6	18	30 / 44
CM2	14	88	6 / 7	1.4	65	46 / 55
CM3	inactive		0	1.2	31	26 / 16
CM4	inactive		0	inactive		0
CM5	5	16	3 / 3	5	42	8 / 9
CM6	5	52	10 / 18	2	19	10 / 18
CM7	3	60	20 / 26	2	32	16 / 21
CM8	4.5	17	4 / 11	inactive		0
CM9	inactive		0	inactive		0
CM10	16	132	8 / 9	3.4	165	49 / 52
CM11	6	129	22 / 39	4	101	25 / 44
CM12	9	138	15 / 29	1.9	79	42 / 81
CM13	3.5	76	22 / 22	1.2	56	47 / 48
CM14	0.7	65	93 / 126	0.6	135	225 / 303
CM15	1	120	120 / 150	0.9	220	244 / 300
Rat D1	0.19	38		0.19	42	

V_{max} and K_m values are the means of at least two experiments, each determined by Lineweaver-Burk analysis of kinetic data.
[1]As a measure for the catalytic efficiency of the mutant enzymes the V_{max}/K_m ratio is presented, either calculated from the measured values or after standardization (Western blotting in Figure 8) for the amount of protein expressed (V_{max}/K_m ratio of measured values / V_{max}/K_m ratio of values after standardization).

With regard to ORD of rT3S the catalytic efficiency of the CM1 to CM13 (except CM12) mutants was lower than that of the cat D1 wt enzyme (Figure 5). This is the consequence of increased K_m values and/or decreased V_{max} values (Table 4). Especially the introduction of the TGMTR insertion (CM5) strongly reduced catalytic efficiency. Single mutants or double mutants (for instance CM6 and CM13) have reduced catalytic efficiency, while the triple mutants CM14 and CM15 have increased catalytic efficiency compared to wt. Similarly as for ORD of rT3 a Phe residue at position 65 is important since the catalytic efficiency of CM14/CM15 is 6-fold higher than CM13. The mutants CM14 and CM15 have similar K_m values as the cat D1 wt enzyme and a 4-fold increased catalytic efficiency. All in all the kinetic characteristics of CM14 and CM15 resemble those of rat/human D1. These mutants still prefer rT3S above rT3 as substrate, but the difference in catalytic efficiency is much smaller than in the wt cat D1 (2-fold vs. 30-fold).

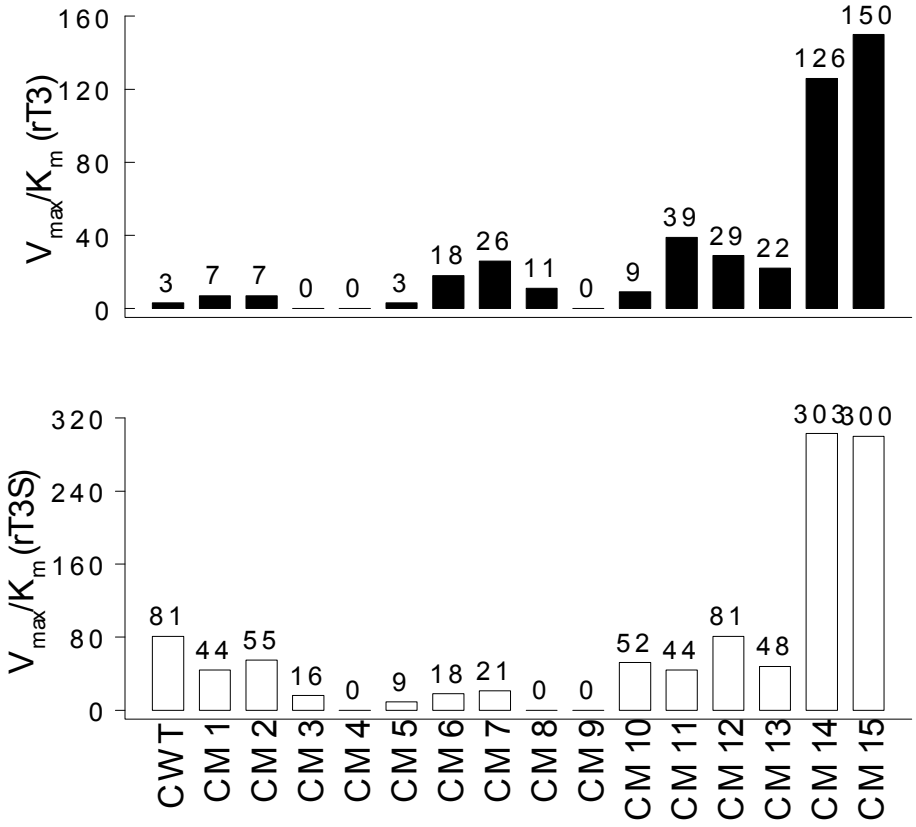

Fig. 5. Kinetic parameters for wt and mutant cat D1 enzymes depicted as V_{max}/K_m ratios (after standardization by Western blotting) for the substrates rT3 and rT3S.

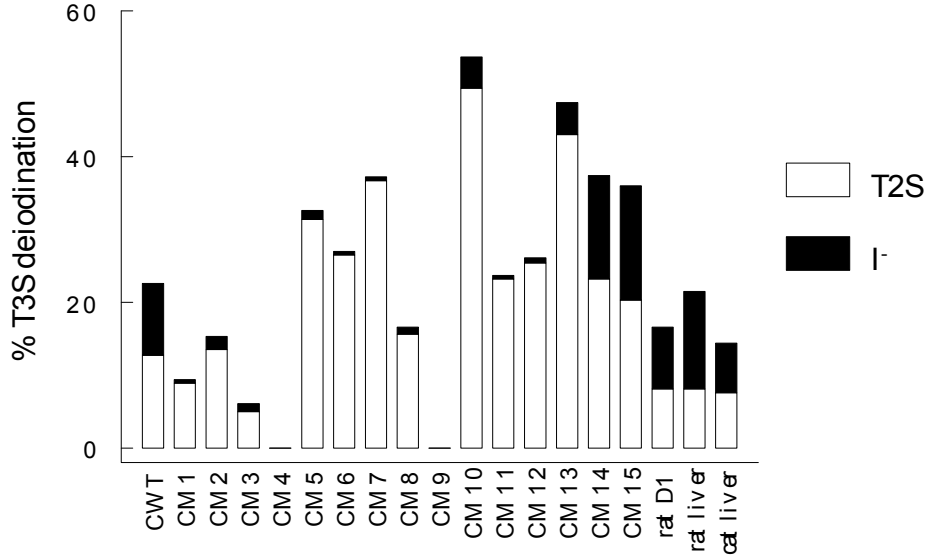

Fig. 6. Inner ring deiodination of T3S (0.1 μM) by wt and mutant cat D1 enzymes in COS cell homogenates (0.14 – 0.17 mg protein/ml). Rat liver and cat liver microsomes (50 μg protein / ml) were analyzed under similar conditions. IRD activity was measured with outer ring ^{125}I-labeled T3S. In this assay IRD activity is the sum of ^{125}I-T2S as well as $^{125}I^-$ formed. The latter is formed by ORD of ^{125}I-T2S.

Site-directed mutagenesis of cat D1 and IRD of T3S

The preferred substrate for IRD by cat D1 is T3S (Figure 1). In this respect cat D1 resembles rat/human D1 for which also IRD of T3 is strongly facilitated by sulfation (7, 42-43). IRD activity was measured with outer ring ^{125}I-labeled T3S. In this assay IRD activity is the sum of ^{125}I-T2S and $^{125}I^-$ formed. The latter is formed by ORD of ^{125}I-T2S. Figure 6 shows the level of the different deiodination products after incubation of 0.1 μM T3S with the D1 mutants. The IRD levels of CWT, CM1, CM2, CM5, CM6, CM7, CM8, CM11, CM12, CM14 and CM15 showed relatively little variation. The accumulation of T2S in incubations with CM1, CM2, CM5, CM6, CM7, CM8, CM11 and CM12 correlates well with their low rT3 and rT3S ORD activity. In incubations with CWT, CM14 and CM15 significant amounts of T2S are further deiodinated (ORD), in accordance with their efficient deiodination of rT3S. The T3S IRD activity of CM10 and CM13 was increased compared to CWT. Not only for ORD but also for IRD a tyrosine at position 66 is important since CM4 and CM9 are inactive. A Phe or Tyr residue at position 65 is not essential since CM5, CM10 and CM13 are more or equally active as CWT, CM3, CM8, CM14 and CM15.

Quantitation of cat D1 mutant enzymes

BrAc[^{125}I]T3 has been used extensively as affinity label for D1, allowing the specific identification of D1 in microsomal fractions of liver and kidney (9-10, 31, 35). Affinity labeling with BrAc[^{125}I]T3 was also used for the quantitation of D1 expression levels by saturation analysis in microsomal preparations and homogenates of transfected cells, allowing the calculation of substrate turnover numbers (9, 19, 22).

Our initial plan was to use affinity labeling for the quantitation of the various cat D1 mutants. However, when equal amounts of homogenate protein were used in affinity labeling experiments up to 6-fold differences in labeling intensity were observed (Figure 7). Especially CM3 (not shown), CM4 (not shown), CM6, CM7, CM11 and CM12 were only weakly labeled (≤ 20% of CWT), precluding saturation analysis for the quantitation of expression levels. In fact, the labeling of CWT was the most intense. The net incorporation of ^{125}I-BrAcT3 in CWT was about 6%. The various mutations likely also influenced the interaction with the BrAcT3-affinity label.

As an alternative to affinity labeling we performed Western blotting using a D1 antiserum directed to the C-terminus of D1 protein (Figure 8). The mutants with the TGMTR insertion, for instance CM5, have a slightly reduced mobility compared to CWT. The

intensity of the protein bands was determined by densitometry and the V_{max}/K_m ratios in Figure 5 were corrected for the measured differences in protein levels. The V_{max} values presented in Table 4 are the measured that is uncorrected values. The Western blotting was repeated two times with homogenates from different transfections, and the expression level of most mutants was close to that of CWT (between 0.7 – 1.1 fold of CWT). The CM6, CM11 and CM12 mutants had reduced expression levels (0.5 – 0.6 fold of CWT), while CM8 and CM9 had a more (< 0.3 – fold of CWT) reduced expression level. The correction of the V_{max}/K_m ratios (Table 4 and Figure 5) for differences in protein expression levels did not cause big changes in the relative catalytic efficiency of the various mutant D1 enzymes, nor did it influence the conclusions drawn.

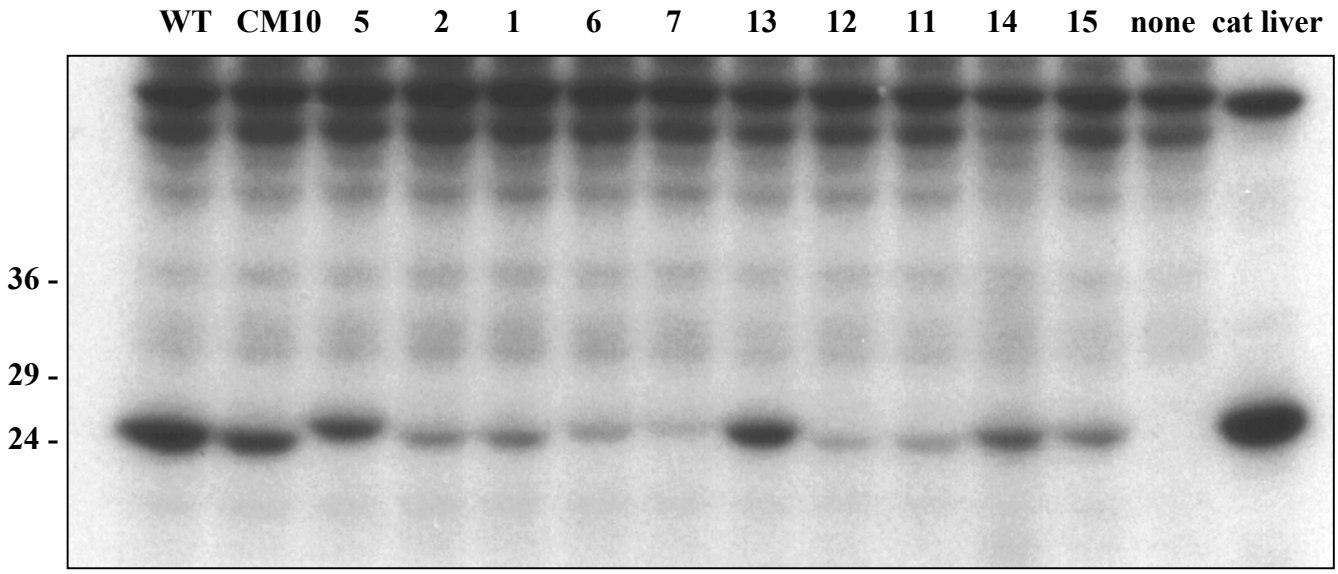

Fig. 7. Labeling patterns obtained by SDS-PAGE and autoradiography after reaction of COS cell homogenates (100 μg protein) containing cat D1 wt or mutant enzymes as indicated with BrAc[^{125}I]T3 in the presence of 10 mM DTT at 37 C. Cat liver microsomes (25 μg protein) were analyzed under similar conditions. Migration distances of molecular mass markers (kilodaltons) are indicated.

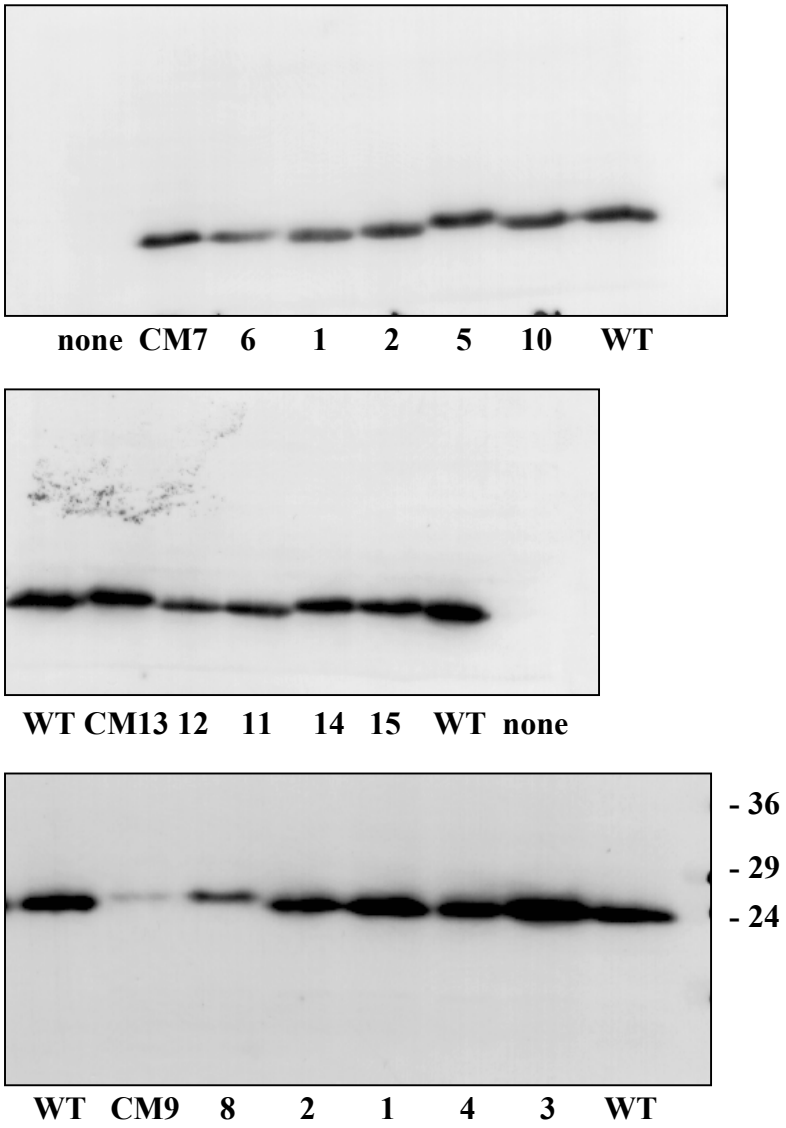

Fig. 8. Western blots of homogenates made from COS cells transiently expressing cat D1 wt or D1 mutant enzymes as indicated. Cells were lysed as described, and 20 – 30 μg of total protein were analyzed on 12 % SDS-PAGE and probed with anti-D1 antibody as described in *Materials and Methods*. Migration distances of molecular mass markers (kilodaltons) are indicated in the lower panel.

DISCUSSION

The aim of the present study was to investigate the molecular basis for the substrate selectivity of cat D1, more in particular the weak interaction with non-sulfated substrates as rT3. By site-directed mutagenesis of cat D1 it was found that a combination of several changes was needed in order to significantly improve the deiodination of rT3. While these

results provide more detailed insight in D1 substrate interaction, an important issue is the physiological role of D1 in cat liver. A substantial fraction of circulating T3 is derived from ORD of T4 by liver D1, at least in rats and humans where detailed investigations were performed (2-5). Due to the presence of an essential SeC residue in D1 it is conceivable that selenium deficiency of rats caused reduction of hepatic D1 activity and increased serum T4 levels (44-46). From the fact that selenium deficiency in kittens caused increased plasma T4 and decreased T3 levels (32) it might be concluded that also in cats (liver) D1 provides a large portion of plasma T3. However, an additional role for D2 in this regard is still possible. We have not detected D2 activity (low K_m and PTU insensitive ORD of T4) in cat liver homogenates.

Reverse T3 appears to be the preferred substrate for D1 in rats and humans, while rT3S is the preferred substrate for cat D1. The role of the deiodination of the biologically inactive rT3 may be the recovery of the trace element iodine. Sulfated iodothyronines as T4S and T3S are deiodinated by human/rat D1 at a significantly faster rate than the corresponding non-sulfated iodothyronines (42-43). The same is true for cat D1 in the sense that deiodination of rT3 and T2 is stimulated by prior sulfation. Since rT3 ORD activity of cat D1 is very low it is likely that in cats metabolism of rT3 occurs via prior sulfation with subsequent deiodination. The balance between the activity of sulfotransferases, sulfatases and deiodinases acting upon iodothyronines is important for the regulation of thyroid hormone levels, especially during fetal development (43, 47-48). We have indeed measured T2 and rT3 sulfation activity in cat liver cytosol (not shown), but we have neither investigated the responsible sulfotranferase(s) nor their kinetic characteristics. Meanwhile, the serum rT3/T4 ratio in cats is elevated compared to humans, indicating reduced efficiency of rT3 metabolism in cats. Apart from D1, other differences between cat and human, for instance in serum binding-protein concentrations and affinities as well as production rates, might influence the plasma rT3 levels.

It is remarkable that a combination of changes that is, the substitution of Phe for Leu at position 65, the insertion of five amino acids (TGMTR) and the mutation of N45R46 to G45E46, is necessary to obtain mutant cat D1 enzymes (CM14 and CM15) with catalytic efficiencies for rT3 ORD comparable to those of rat/human D1. In fact, the combination of the changes causes a 4-fold further increase in the efficiency for ORD of rT3S compared to cat D1 wt. Each of the changes alone or even combinations of two changes have either no or only a small impact on rT3 ORD, while for rT3S ORD they reduce catalytic efficiency. The substitution of Phe for Leu65 causes a big increase in catalytic efficiency both for rT3 and

rT3S ORD. This is most clear in the context of the other two changes, i.e. the TGMTR insertion and the GE for NR substitution (compare CM13 with CM14). In this regard our results are in line with previous studies on dog D1 that suggested the interaction of the inner ring of rT3 with the aromatic ring of Phe65 (22, 30). The substitution of Tyr for Leu65 as such is detrimental or causes only a small increase in activity in combination with the TGMTR insertion. We did not test the Leu65Tyr mutation in the context of the TGMTR insertion and the NR to GE change. Nevertheless, it is likely that this mutant would have improved rT3 ORD activity compared to cat D1 wt although probably not to the same extent as mutants with a Phe at position 65. Recently, D1 from the killifish *Fundulus heteroclitus* was cloned (25) and it contains a Tyr residue at position 65 (Y65G66) in combination with the 'insertion' VTMTQ and G45E46 (Figure 3). *Fundulus heteroclitus* D1 has a K_m for ORD of rT3 in the same range as rat/human D1 (0.12 μM), but no comparative data on catalytic efficiencies (V_{max}/K_m ratio's) for rT3 and rT3S are available. The substitution of Leu for Tyr66 completely inactivates cat D1 (CM4 and CM9). This might indicate that in wt cat D1 rT3 and rT3S interact with Tyr 66 in the absence of Phe at position 65.

The insertion of TGMTR (48 – 52) as such in cat D1 (CM5) does not improve ORD of rT3 and greatly decreases ORD of rT3S. However, the TGMTR insertion is important in the context of a Phe at residue 65 and the NR to GE change. The ORD efficiency of CM14/CM15 is about 5-fold higher than CM11/CM12 (no TGMTR insertion) both for rT3 and rT3S. Either the positioning of Phe65 towards the inner-ring of rT3 and rT3S is improved by this insertion and/or the positioning of the outer-ring towards the catalytic center (SeC) is improved. From the fact that especially for rT3 the increased V_{max}/K_m ratio is mainly caused by a decrease of the K_m value it might be concluded that the main effect is improved interaction of Phe65 with rT3. Our results are in contrast to the study of Toyoda et al (30) who found that the TGMTR insertion in dog D1 does not improve ORD of rT3 while it inhibits ORD of T2S, and therefore Bianco et al (3) concluded that 'these five amino acids are not critical to D1 function'. However, Toyoda et al (30) did not test the TGMTR insertion in the context of the substitution of Phe for Leu at position 65 and the GE (45-46) substitution for NR but only as such in wt dog D1. They may, thus, have overestimated the importance of the substitution of Phe for Leu65 in dog D1. Our results with cat D1 show that the three changes as such cause only small improvements in rT3 ORD, but that the combination of all three changes is synergistic and that each change is necessary.

An intriguing property of wt cat D1 is the facilitated deiodination of rT3S. Both rT3 and rT3S are only deiodinated in the outer ring, and therefore both substrates bind in such a way

that the iodines of the outer ring are in close proximity to the catalytic center, that is the SeC residue. The negatively charged sulfate group of rT3S might interact with the positively charged side group of a basic amino acid (Lys, Arg), thereby stabilizing the interaction with D1. Initially, we thought that R46, which is unique for cat D1 at that position, fulfills this role. However, the mutation R46E did not inhibit rT3S deiodination. Of course, another basic amino acid residue in D1 might be involved. Alternatively, conformational differences between rT3 and rT3S may influence interaction with D1. Crystallographic data support a so-called antiskewed conformation for rT3 (49-50), but as far as we know no structural data are available for rT3S.

The IRD activity with T3S showed relatively little variation with the different constructs, especially if one compares the IRD activity of CWT and CM14/CM15. In other words, none of the mutated residues in the 40 – 70 residue region involved in rT3 and rT3S interaction / ORD are essential for T3S interaction and IRD. The fact that liver microsomal fractions catalyze both ORD and IRD has always been difficult to understand, however since the cloning of D1 it is certain that this involves a single enzyme (6-8, 51-53). The fact that D1 catalyzes ORD and IRD suggests different orientations of substrate binding within a single site, so that either the iodines of the inner ring or of the outer ring are in close proximity to the catalytic center. Alternatively, two different substrate-binding sites connected with deiodination might exist, one for ORD (rT3, rT3S) and one for IRD (T3S). On first sight our data support the two substrate binding site model, consisting of a site which binds substrates with two iodines in the outer ring and involved in ORD and one site which binds substrates with two iodines in the inner ring and involved in IRD. This could explain why T4 undergoes ORD as well as IRD. So T4, with two iodines in both rings, would have to interact with both sites. A problem with this model is the shift in deiodination preference of T4 upon sulfation. IRD of T4 by rat/human D1 is strongly facilitated following its sulfation, whereas ORD of T4S is undetectable (43, 54). This would imply that the binding site connected with ORD would accept rT3S and T2S but not T4S, which is difficult to explain. All in all the most simple model is to assume that D1 has a single substrate binding site with limited substrate specificity and that the various substrates bind in orientations which either favor ORD or IRD. More detailed insights in D1 structure-function relationships must come from the three-dimensional structure when this is resolved by crystallographic studies. Unfortunately, these studies are greatly hampered by the difficulties encountered with overexpressing this membrane-integrated enzyme in a soluble active form.

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CHAPTER 3

CHARACTERISTICS AND THYROID STATE-DEPENDENT REGULATION OF IODOTHYRONINE DEIODINASES IN PIGS

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The porcine iodothyronine deiodinase coding sequences have been submitted to the GenBank database under accession numbers AY533206 (type I iodothyronine deiodinase), AY533207 (type II iodothyronine deiodinase) and AY533208 (type III iodothyronine deiodinase).

ABSTRACT

Three iodothyronine deiodinases (D1-D3) regulate local and systemic availability of thyroid hormone. D1 and D2 activate the prohormone thyroxine (T4) to the thyromimetic 3,3',5-triiodothyronine (T3), and D3 inactivates T4 and T3 to 3,3',5'-triiodothyronine (rT3) and 3,3'-diiodothyronine (3,3'-T2), respectively. The expression of the three deiodinases is tightly regulated with regard to developmental stage and cell type in order to provide fine-tuning of T3 supply to target cells. Most studies regarding distribution and regulation of deiodinases have been carried out in rodents. However, in different respects rodents do not seem to be the optimal experimental model for human thyroid hormone physiology. For instance, D2 expression has been observed in human thyroid and skeletal muscle, but not in these tissues in rodents. In this study we have explored the pig as an alternative model. Porcine D1, D2 and D3 were cloned by RT-PCR, and their catalytic properties were shown to be virtually identical to those reported for human and rodent

deiodinases. The tissue distribution of deiodinases was studied in normal pigs and in pigs made hypothyroid by methimazole treatment or in pigs made hyperthyroid by T4 treatment. D1 activity in liver and kidney was increased in T4-treated pigs. D2 activities in cerebrum and pituitary were decreased after T4 treatment and strongly increased after methimazole treatment. Remarkably, D2 activity in thyroid and skeletal muscle was induced in hypothyroid pigs. Significant expression of D3 was observed in cerebrum, and was positively regulated by thyroid state. In conclusion, the pig appears to be a valuable model for human thyroid hormone physiology. Especially, the expression of D2 activity in thyroid and skeletal muscle is of interest for studies on the importance of this enzyme in (hypothyroid) humans.

INTRODUCTION

Thyroid hormone is essential for growth, development and regulation of energy metabolism (1-3). Particularly well known is the critical role of thyroid hormone in development and function of the central nervous system (4). Thyroid hormone is produced by the thyroid in the form of the biologically inactive precursor T4. The principal bioactive form of the hormone is T3. In humans, only about 20% of T3 is secreted by the thyroid; most circulating T3 is derived from outer ring deiodination (ORD) of T4 in peripheral tissues (1-3). Both T4 and T3 undergo inner ring deiodination (IRD) to metabolites which do not interact with T3 receptors, rT3 and 3,3'-diiodothyronine (3,3'-T2), respectively. Thus, ORD is regarded as an activating pathway and IRD as an inactivating pathway. ORD is also the main pathway for the metabolism of rT3, representing another route for the generation of 3,3'-T2. Three iodothyronine deiodinases are involved in the deiodination of iodothyronines, *i.e.* D1-D3 (1-3).

In humans and rodents, D1 is located primarily in liver, kidney and thyroid (5-12). Lower D1 activities are expressed in other tissues, including rat anterior pituitary (13). Although D1 has both ORD and IRD activities, it appears particularly important for the generation of plasma T3 and clearance of plasma rT3 (1, 3). ORD of rT3 is the most efficient reaction catalyzed by D1, while IRD of both T4 and T3 are strongly accelerated by sulfation of these iodothyronines (1). Michaelis Menten constant (K_m) values for

substrates of D1 are in the micromolar range. The enzyme is potently inhibited by the thyrostatic drug 6-propyl-2-thiouracil (PTU) (1-3, 14). D1 activity is positively regulated by T3, reflecting regulation of D1 expression by T3 at the pretranslational level (15). D1 activity in the thyroid is stimulated by TSH and in FRTL-5 rat thyroid cells by thyroid-stimulating antibodies from patients with Graves' disease (16).

In humans, D2 activity is found in brain, anterior pituitary, placenta, thyroid and skeletal muscle, and D2 mRNA has also been detected in the human heart (3-4, 17-21). In rodents D2 is also expressed in brown adipose tissue (22-23). D2 has only ORD activity, preferring T4 to rT3 as the substrate, with apparent K_m values in the nanomolar range (17, 20). In general, D2 activity is increased in hypothyroidism and decreased in hyperthyroidism. Both pre- and posttranslational mechanisms are involved in the regulation of D2 expression by thyroid state, with distinct roles for T3, and for T4 and rT3, respectively (24-28). Although perhaps D2 in skeletal muscle may contribute to circulating T3, the enzyme is particularly important for local T3 production in brain and anterior pituitary (3, 29).

In human and rodents, D3 is located in brain, placenta, pregnant uterus, and fetal tissues (7, 30-33). D3 has only IRD activity, and is thus important for the inactivation of thyroid hormone. It shows preference for T3 over T4 as the substrate, with apparent K_m values in the nanomolar range (33). The high D3 activity in placenta, pregnant uterus and different fetal tissues seems to serve the purpose of protecting the fetus against undue exposure to active thyroid hormone that may be detrimental for the development of different tissues, in particular the brain (4, 31-32). In brain, D3 activity is increased in hyperthyroidism and decreased in hypothyroidism but the mechanism of this regulation remains to be established (34-35).

Most studies regarding distribution and regulation of deiodinases have been carried out in rats. However, in different respects the rat does not seem to be the optimal experimental model for human thyroid hormone physiology. This is most obvious for the fetal and neonatal development of the tissues, which follow different patterns relative to the time of birth in humans *versus* rats. Little D3 is expressed in rat liver at any stage of development, whereas high D3 activity is detected in fetal human liver or in liver of severely sick patients (7, 31, 36-37). Furthermore, D2 expression has been observed in

human thyroid and skeletal muscle, but not in these tissues in the rat (17-21, 38-39). Therefore, in order to investigate thyroid hormone metabolism in different tissues such as thyroid and skeletal muscle, we explored the pig as an alternative animal model. Iodothyronine deiodinase activities (D1, D2 and D3) have been described in liver, kidney and placenta of pig fetuses as well as in neonatal pigs (40-41). Our studies involved the investigation of the molecular characteristics, the tissue distribution, as well as the thyroid state-dependent regulation of the three iodothyronine deiodinases in pigs.

MATERIALS AND METHODS

Animals and treatment

Two to three months old male and female Yorkshire x Landrace pigs were studied. All experiments were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH publication 86-23, revised 1996) and with prior approval of the Erasmus Medical Center Animal Care Committee. Two pigs (one male, one female) were made hypothyroid by oral treatment with methimazole at a dose of 5 mg/kg/day. Two pigs (one male, one female) were made hyperthyroid by oral treatment with T4 at a dose of 50 μg/kg/day. Both T4 and methimazole were administered in capsules (made by the Erasmus Medical Center Pharmacy with lactose as sweetener), which were mixed with the food. Four untreated pigs were included as controls. Once a week, body weight was measured, and animals were sedated by i.m. injection of 10 mg/kg ketamine and 0.5 mg/kg midazolam; blood was collected for measurement of plasma T4 and T3 levels to ensure adequacy of the methimazole and T4 doses. At the end of the 4-week period, pigs were sedated with ketamine (20 mg/kg i.m.) and midazolam (0.5 mg/kg i.m.), anesthetized with pentobarbital (20 mg/kg i.v.), intubated, and ventilated with a mixture of O_2 and N_2. Fluid-filled catheters (8 French) were inserted into the jugular vein for infusion of pentobarbital (10-15 mg/kg per h i.v.), and via a femoral artery into the aorta for measurement of arterial blood pressure. A high-fidelity microtipped pressure-transducer was advanced via a carotid artery into the left ventricle (LV) for measurement of LV pressure and its first derivative LVdP/dt, while a Swan-Ganz catheter was inserted via a femoral vein and advanced into the pulmonary artery for measurement of cardiac output according to the Fick method (42).

All measurements were done in duplicate, after which animals underwent a midsternal thoracotomy, and the heart, liver, kidneys, skeletal muscle (*Musculus iliopsoas*), thyroid, pituitary, and cerebrum were isolated, collected in liquid N_2, and stored at –80 C for further analysis.

Materials

Nonradioactive iodothyronines were obtained from Henning Berlin (Berlin, Germany); [3'-^{125}I]T3 and [3',5'-^{125}I]T4 were obtained from Amersham (Little Chalfont, UK); and [3',5'-^{125}I]rT3 was prepared in our laboratory by radioiodination of 3,3'-T2 as described previously (43). [^{125}I]T3 could be used without further purification, but [^{125}I]T4 and [^{125}I]rT3 were purified on Sephadex LH-20 before each experiment. ^{125}I-labeled and unlabeled T3 sulfate (T3S) and 3,3'-T2 sulfate (3,3'-T2S) were prepared as previously reported (44). Dithiothreitol (DTT), PTU, methimazole, T4 (for treatment of pigs), goldthioglucose (GTG), iodoacetate (IAc), and β-mercaptoethanol were obtained from Sigma (St.Louis, MO); electrophoresis grade SDS-PAGE reagents, protein markers and protein assay reagent from Bio-Rad (Richmond, IL); Sephadex LH-20 and DEAE-dextran from Pharmacia (Uppsala, Sweden); TRIzol reagent, synthetic oligonucleotides, rTaq polymerase, cell culture medium and fetal bovine serum from GIBCO BRL (Breda, The Netherlands); oligo(dT), and random hexamer primers, dNTP, RNAse inhibitor, M-MLV reverse transcriptase, *HindIII*, pGEM-T vector from Promega (Madison, WI).

Cloning of porcine deiodinases and construction of expression vectors

Total RNA was isolated from different porcine tissues (liver for D1 cloning, pituitary for D2 cloning and cerebrum for D3 cloning) using TRIzol reagent. cDNA was obtained by reverse transcription of 5 μg total RNA using random hexamer primers as well as oligo(dT) primers and M-MLV reverse transcriptase. Initially, oligonucleotide primers homologous to sequences surrounding the start or stop codons of human/mouse/rat deiodinases were designed (Table 1) and used for PCR reactions with the respective cDNA samples. For D1, the sense and antisense primers contain the start and stop codon, respectively. For D2, the sense primer is located just upstream of the start codon while the antisense primer contains the stop codon. Unfortunately, for D3 it was necessary to locate

the sense primer just downstream of the start codon and the antisense primer was located in the SECIS element (selenocysteine insertion sequence element). The PCR products obtained (about 750 bp for D1, about 850 bp for D2 and about 1400 bp for D3) were cloned in the pGEM-T vector and sequenced in both directions.

Table 1. Oligonucleotide primers used for cloning of porcine deiodinases by RT-PCR.

Deiodinase	Sense primer	Antisense primer
D1	5'TCTGGCTTTGCCGAGATG	5'GAGGTATCTGTCCAGATTAAC
D2	5'AGAGCGCACAAGGGAACTGAC	5'GTTTTCTTTTATCTCTTGCTG
D3	5'CTGCTGCTTCACTCCTTGAG	5'AGTTAGAGATAGTTCGTCAT

Start and stop codons, if present, are underlined. The cDNA for the human D2 coding region contains two in-frame SeC (TGA) codons (17, 20, 50). The 3'TGA is seven codons 5' to a universal stop codon (TAA). In the D2 antisense primer used here the putative 3'TGA is changed to a universal stop codon TTA. In human D2 the second SeC and the following seven amino acid residues are not critical for its function (50).

Because a SECIS element is required for incorporation of SeC in selenoproteins, we prepared chimeric constructs in which the porcine deiodinase coding sequence cDNAs were inserted 5' to the SECIS element of the rat D1 gene. Expression plasmids were constructed by PCR of the pig D1 (pD1), pD2 and pD3 coding sequence (CDS) in the above mentioned pGEM-T plasmids using primers located over or close to the translation start and stop codons and adapted with *HindIII* restriction sites (Table 2). For the D3 expression vector a start codon and three amino acids were introduced (MLHS). These PCR products were cloned into the pGEM-T vector, excised with *HindIII*, and subcloned into the *HindIII* sites of the rD1SECIS-pcDNA3 plasmid. The latter was obtained by *HindIII* digestion of the G21-pcDNA3 plasmid kindly provided by Dr. P. Reed Larsen (Harvard Medical School, Boston), and contains part of the 3'-UTR of rat D1, including the SECIS element. The inserts of these plasmids (pD1/2/3-rD1SECIS-pcDNA3) were sequenced in both directions.

Table 2. Oligonucleotide primers used for the construction of deiodinase expression vectors.

Deiodinase		Oligonucleotide primer
D1	sense	5'C<u>AAGCTT</u>*GCCACC**ATG**G*AGCTGCCCCTGCCA
	antisense	5'C<u>AAGCTT</u>**TTA**ACTGTGGAGCTTTTC
D2	sense	5'C<u>AAGCTT</u>AGAGCGCACAAGGGAACTGAC
	antisense	5'C<u>AAGCTT</u>GTTTTCTT**TTA**TCTCTT
D3	sense	5'C<u>AAGCTT</u>ACC**ATG**CTCCACTCCCTGCTGCTTCACTCCTTGAGG
	antisense	5'C<u>AAGCTT</u>**TTA**CACTCGACGGGGCTG

Hind III restriction sites are underlined, and the Kozak consensus translational initiation sequence, introduced in the D1 expression vector is shown in Italics. Start and stop codons are typed in bold. The sequence encoding the four extra amino acids (MLHS) introduced at the D3 enzyme N-terminus are shown in lower case.

Native deiodinases and expression of recombinant deiodinases

Tissues were homogenized and microsomal fractions were prepared by differential centrifugation as previously described (8) in 100 mM phosphate buffer (pH 7.2) and 2 mM EDTA (PE), containing 1 mM DTT (PED1). Protein concentrations were determined using the Bio-Rad protein assay reagent and BSA as the standard. Aliquots of homogenates and microsomes were snap-frozen on dry ice/ethanol, and stored at – 80 C until further analysis.

Recombinant deiodinases were expressed in COS-1 cells after DEAE-dextran mediated transfection of expression plasmids as described (45-46). Two days after transfection, the cells were rinsed with PBS, collected in 0.25 ml 0.1 M phosphate buffer (pH 7.2), 2 mM EDTA, and 10 mM DTT (PED10), sonicated, aliquoted, snap-frozen on dry-ice/ethanol, and stored at – 80 C.

Deiodinase enzyme activity measurements

Deiodinase activities of native and recombinant enzyme preparations were analyzed either by quantitation of radioiodide released by ORD of outer ring ^{125}I-labeled rT3 (D1) or T4 (D2), or by analysis of radioactive 3,3'-T2 generated by IRD of outer ring ^{125}I-labeled T3 (D3) by high-performance liquid chromatography (HPLC).

D1 assay. Appropriate amounts of liver homogenates or microsomal fractions were incubated in triplicate for 60 min at 37 C with 100000 cpm [3',5'-^{125}I]rT3 and varying amounts of unlabelled iodothyronines in 0.1 ml PE buffer containing 10 mM DTT (PED10). Blank incubations were carried out in the absence of microsomal protein (buffer blank). Reactions were stopped by addition at 4 C of 0.1 ml 5% (wt/vol) BSA in water followed by addition of 0.5 ml 10% (wt/vol) trichloroacetic acid in water. After pelleting of the precipitated [^{125}I]iodothyronines, [^{125}I]iodide was further isolated from the supernatant on LH-20 minicolumns, equilibrated and eluted with 0.1 M HCl (8). In the case of COS cell homogenates 50 – 100 μg protein was incubated in the same manner. Total deiodination was corrected for non-enzymatic deiodination in blank incubations with homogenate from non-transfected COS cells (< 5 % of total deiodination).

Inner ring deiodination was studied with outer ring labeled T3S ([3'-^{125}I]T3S). In this assay IRD activity is the sum of [3'-^{125}I]T2S, [3'-^{125}I]T1S as well as $^{125}I^-$ formed. The latter is formed by outer ring deiodination of ^{125}I-T2S, while ^{125}I-T1S is formed by IRD of T2S. Liver microsomal fractions (10 – 250 μg protein/ml) were incubated for 60 min at 37C with 10 nM ^{125}I-T3S (200000 cpm) in 0.1 ml PED10. The reaction was stopped by the addition of methanol (1:1) on ice, and the reaction mixtures were analyzed by reverse-phase HPLC as previously described (47).

D2 assay. Appropriate amounts of homogenates or microsomal fractions (only homogenate for pituitary) were incubated for 60 min at 37 C with 1 nM (100000 cpm) [3',5'-^{125}I]T4 in the presence of 100 nM unlabeled T3 to inhibit D3 activity, and in the absence or presence of 100 nM unlabeled T4 to saturate D2, in 0.1 ml PE buffer containing 25 mM DTT (PED25). Release of $^{125}I^-$ was determined and corrected for nonenzymatic deiodination as described above. The difference in fractional deiodination between incubations with 1 and 100 nM T4 represented low-K_m D2 activity. In additional experiments 1 nM labeled T4 was incubated with microsomal fractions or COS cell homogenates in the presence of varying amounts of unlabeled iodothyronines.

D3 assay. Appropriate amounts of homogenates or microsomal fractions were incubated in triplicate for 1-4 h at 37 C with 1 nM (200000 cpm) [^{125}I]T3 in the absence or presence of 100 nM unlabeled T3 to saturate D3 in 0.1 ml PE buffer containing 50 mM DTT (PED50). The reactions were stopped by addition of 0.1 ml ice-cold MeOH. After

centrifugation, 0.1 ml of the supernatant was mixed with 0.1 ml 0.02 M ammonium acetate (pH 4), and 0.1 ml of the mixture was applied to a 250x4.6 mm Symmetry C18 column connected to an Alliance HPLC system (Waters, Etten-Leur, The Netherlands), and eluted isocratically with a mixture of acetonitrile and 0.02 M ammonium acetate pH 4 (33:67, vol/vol) at a flow of 1.2 ml/min. Radioactivity in the eluate was monitored on-line using a Radiomatic A-500 flow scintillation detector (Packard, Meriden, CT). Conversion of labeled T3 to radioactive 3,3'-T2 was corrected for nonenzymatic deiodination as observed in blanks incubated in the absence of microsomal protein (buffer blank). The difference in fractional deiodination between incubations with 1 and 100 nM T3 represented low-K_m D3 activity. In additional experiments 1 nM labeled T3 was incubated with microsomal fractions or COS cell homogenates in the presence of varying amounts of unlabelled iodothyronines.

Western blotting with D1 antiserum

Polyclonal antisera were raised in rabbits against the keyhole limpet hemocyanin (KLH) conjugate of the synthetic peptide (C)NPEEVRAVLEKLHS (human D1 amino acid residues 236-249). This antiserum cross-reacts with porcine D1 (47).

Microsomal preparations from porcine liver and kidney (2.5 – 25 μg microsomal protein and BSA to 25 μg total protein) were separated on 12% SDS-PAGE gels in the Mini-Protean III cel (Bio-Rad Laboratories, Hercules, CA) according to manufacturer's instructions. After electrophoresis the proteins were blotted to nitrocellulose membrane (Hybond ECL, Amersham Pharmacia Biotech), incubated with primary antiserum (1:500) and subsequently incubated with peroxidase-conjugated secondary antibody as described previously (46).

Affinity labeling of D1 with N-bromoacetyl-[^{125}I]T3

BrAc[^{125}I]T3 (1500 mCi/μmol) was synthesized as described (12, 47), and HPLC analysis demonstrated that the purity was at least 85% with unreacted [^{125}I]T3 as the main contaminant. Solutions of BrAc[^{125}I]T3 (100,000 cpm, 0.03 pmol) in ethanol were pipetted into microcentrifuge tubes, and the solvent was evaporated by a stream of nitrogen. After addition of 50 μl PED10 and vortexing, the liver or kidney microsomal fractions (100 μg

protein) were added in a total volume of 25 µl PED10. COS cel homogenates with porcine or rat D1 protein were used as controls. The mixtures were incubated for 15 min at 37 C. Reactions were terminated by addition of SDS-PAGE gel-loading buffer, and samples were analyzed by SDS-PAGE (12% gel) followed by autoradiography to Kodak BioMax MS film (Eastman Kodak, Rochester, NY).

Hormone measurements

Plasma T4 and T3 levels were determined by RIA (48). Radiolabeled iodothyronines were obtained from Amersham-Pharmacia Biotech, and T4 and T3 antisera were produced previously (48). The sample volume was 50 µl for T4 and 25 µl for T3. The incubation volume was 0.5 ml RIA buffer (0.06 M barbital, 0.15 M NaCl, 0.1 % BSA, and 0.6 g/l 8-anilino-1-naphthalenesulfonic acid, pH 8.6). Mixtures were incubated in duplicate overnight at 4 C, and antibody-bound radioactivity was precipitated using Sac-Cel cellulose-coupled second antibody (IDS, Boldon, UK). The lower limit of detection was 2 nmol/L for T4 and 0.08 nmol/L for T3, and all plasma samples were measured in the same assay. The free fractions of plasma T4 and T3 were determined by equilibrium dialysis (49), and multiplied with the total T4 and T3 levels for calculation of the FT4 and FT3 concentrations.

RESULTS

Cloning of porcine iodothyronine deiodinases

The coding sequences of the porcine deiodinases were cloned by RT-PCR on total RNA isolated from tissues with particularly high expression of these enzymes, using primers derived from the nucleotide sequences of the human deiodinases. Pig D1 (pD1) was cloned from liver, pig D2 (pD2) from pituitary, and pig D3 (pD3) from cerebrum. Initial attempts were carried out using forward/sense primers representing the 5'-flanking region and reverse/antisense primers representing the 3'-flanking region of the human deiodinase coding sequences (see Table 1). This approach produced cDNA clones of the coding sequences of pD1 and pD2. For pD1 the primers used overlap the start and stop codon, while for pD2 the forward/sense primer was located 20 bp upstream of the start

codon. Unfortunately, for the cloning of pD3 this approach was not successful. The forward/sense primer had to be chosen downstream of the human D3 start codon, while the reverse/antisense primer was located in the SECIS element. The deduced amino acid sequences are presented in Figure 1 and are aligned with corresponding sequences of human (h) and rat (r) deiodinases. The amino acid sequence of pD1 shows 85% identity with hD1, 78% with rD1, and 77% with mouse (m) D1. The amino acid sequence of pD2 has 92% identity with hD2, 90% with rD2, and 90% with mD2. Finally, the amino acid sequence of pD3 is 94% identical with hD3, 91% with rD3, and 89% with mD3. Deiodinases contain a selenocysteine (SeC) residue in the catalytic center, which is essential for catalytic activity (2-3, 45-46). The core catalytic center of about 15 amino acid residues around the SeC residue is completely conserved in the porcine deiodinases, including the typical Cys to Ala substitution two residues N-terminal of the SeC residue in D2 enzymes (45).

Expression vectors were made as described in *Materials and Methods* using the oligonucleotide primers in Table 2. The expression vectors contained the rat D1 SECIS element downstream of the coding sequences. In the pD3 expression vector the start codon and three amino acid residues (MLHS), were introduced (see Table 2 and Figure 1).

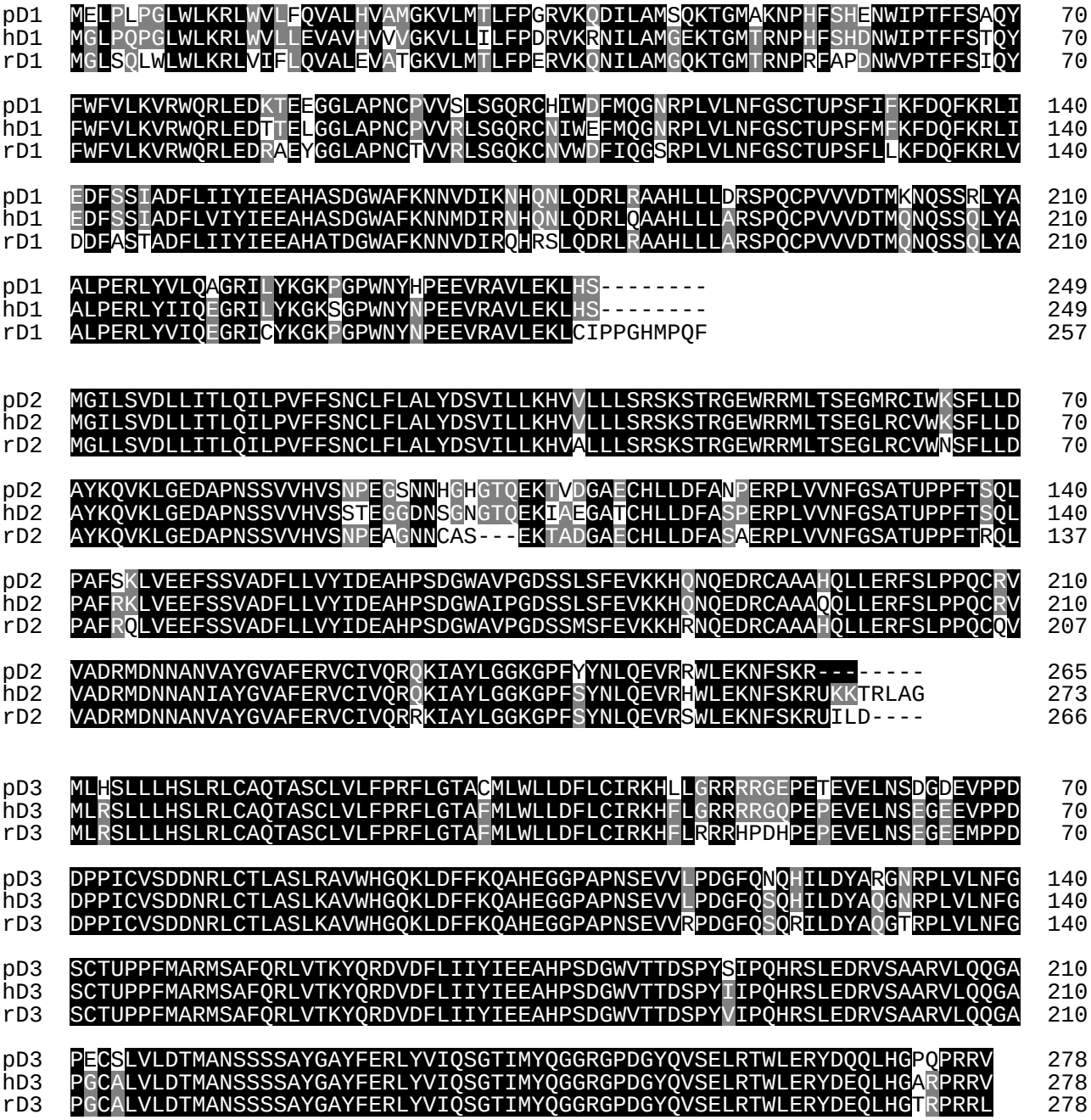


Fig. 1. Comparison of human, porcine and rodent deiodinases. Alignment of the deduced amino acid sequences of human (h), porcine (p), and rat (r) D1, D2, and D3 iodothyronine deiodinases. The selenocysteine (SeC) residue in the catalytic center is indicated by U.

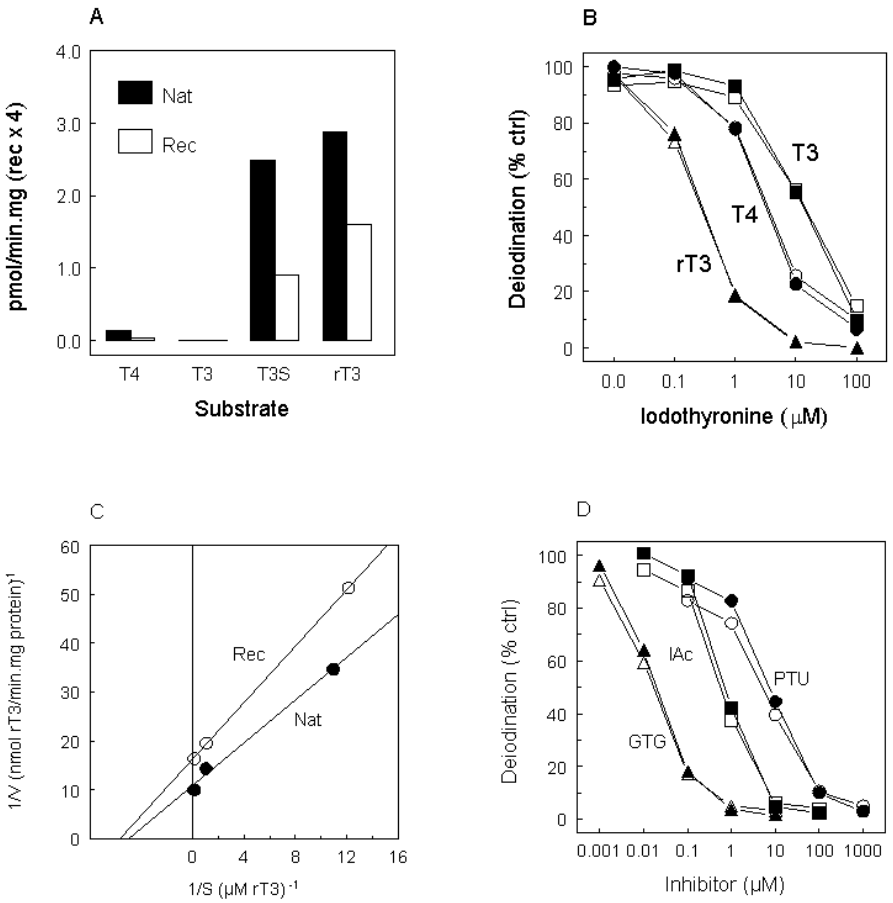


Fig. 2. Characterization of pig D1 enzyme.
A. ORD of T4, T3 and rT3 as well as IRD of T3S by recombinant D1 expressed in COS cells (open bars) and native D1 in porcine liver microsomes (closed bars). Assay mixtures contained 10 nM ^{125}I-substrate (100000 cpm), 10 mM DTT, and 0.3 (lysate) or 0.05 (liver microsomes) mg protein/ml and were incubated for 60 min at 37C.
B. Inhibition of the ORD of ^{125}I-rT3 by recombinant D1 (open symbols) or native D1 enzyme (closed symbols) by 0.1 – 100 μM unlabeled rT3, T4, or T3. Assay mixtures contained 10 nM ^{125}I-rT3 (100000 cpm), 10 mM DTT, and 0.1 (lysate) or 0.01 (liver microsomes) mg protein/ml, and were incubated for 60 min at 37C.
C. Double-reciprocal plot of the rate of rT3 deiodination catalyzed by recombinant D1 enzyme (open symbols) and native D1 enzyme (closed symbols).
D. Inhibition of the ORD of ^{125}I-rT3 by recombinant D1 (open symbols) and native D1 (closed symbols) by increasing concentrations of GTG (0.001 – 10 μM), IAc (0.01 – 100 μM), or PTU (0.1 – 1000 μM). Assay mixtures contained 10 nM ^{125}I-rT3 (100000 cpm), 10 mM DTT, and 0.1 (lysate) or 0.01 (liver microsomes) mg protein/ml and were incubated for 60 min at 37C.

Catalytic characterization of native and recombinant porcine deiodinases

Deiodinase activities were studied under initial reaction rate conditions, with conversion rates being linear with protein concentration and incubation time. Pig liver microsomes were used as a source of native pD1, and its activity was compared with that of recombinant pD1 expressed in COS-1 cells (Fig. 2). Native and recombinant pD1 showed 1) ORD activity with T4 and rT3, and IRD activity with T3S (Fig. 2A); 2) much higher rates for ORD of rT3 than of T4 (Fig. 2A); 3) much higher rates for IRD of T3S than of nonsulfated T3 (Fig. 2A); 4) equal dose-dependent inhibition of the ORD of [^{125}I]rT3 by unlabeled iodothyronines with approximate IC_{50} values of 0.2 μM rT3, 2 μM T4 and 10 μM T3 (Fig. 2B); 5) similar apparent K_m values for rT3 (≈0.2 μM) as determined by Lineweaver-Burk analysis (Fig. 2C; Table 3); and 6) identical sensitivity to well-known D1 inhibitors with approximate IC_{50} values of 0.02 μM GTG, 1 μM IAc, and 10 μM PTU (Fig. 2D). The potency of PTU inhibition increased 10-fold if the rT3 substrate concentration was increased from 10 to 100 nM (data not shown), in agreement with the uncompetitive nature of PTU inhibition (2, 14).

These findings demonstrate that native and recombinant pD1 have identical catalytic properties, substrate specificities and inhibitor sensitivities. Moreover, these characteristics are in good agreement with previous studies of human and rat D1 (2, 8, 11). However, when the deiodination of [3'-^{125}I]T3S by porcine liver microsomes was studied in comparison with rat and human liver microsomes, a remarkable difference in product formation was observed (Fig. 3). T3S was very rapidly deiodinated by all three D1 enzymes through IRD to 3,3'-T2S as the initial product (IRD of outer ring labeled [3'-^{125}I]T3S gives [3'-^{125}I]T2S). Although this intermediate was completely converted by rat D1 via ORD (only $^{125}I^-$ produced), some IRD to [3'-^{125}I]T1S by human D1 was observed, whereas 3,3'-T2S was converted by porcine D1 to almost equal extents via ORD and IRD (note that $^{125}I^-$ is produced by ORD of 3,3'-T2S and 3'-T1S). These differences in deiodination of 3,3'-T2S by pig, human and rat D1 via ORD or IRD were also documented using 3,[3'-^{125}I]T2S as the substrate (data not shown).

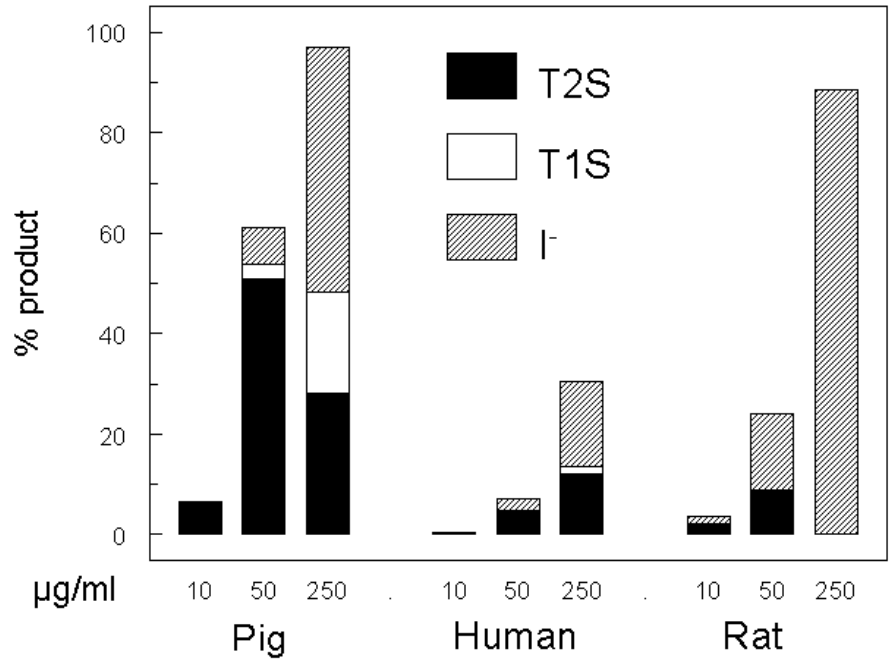


Fig. 3. Deiodination (IRD and ORD) of sulfated iodothyronines by human, porcine and rat D1 enzyme. Liver microsomal fractions (10 – 250 µg protein/ml) were incubated for 60 min at 37 C with 10 nM [3'-^{125}I]T3S (200000 cpm) in 0.1 ml PED10 buffer. The reaction was stopped by the addition of methanol, and the mixture was analyzed by reverse-phase HPLC as described (47). In this assay IRD activity is the sum of ^{125}I-T2S, ^{125}I-T1S as well as $^{125}I^-$ formed. The latter is formed by ORD of ^{125}I-T2S, while ^{125}I-T1S is formed by IRD of T2S. Whereas porcine D1 catalyzes both ORD and IRD of T2S, human and rat D1 cause only ORD of T2S.

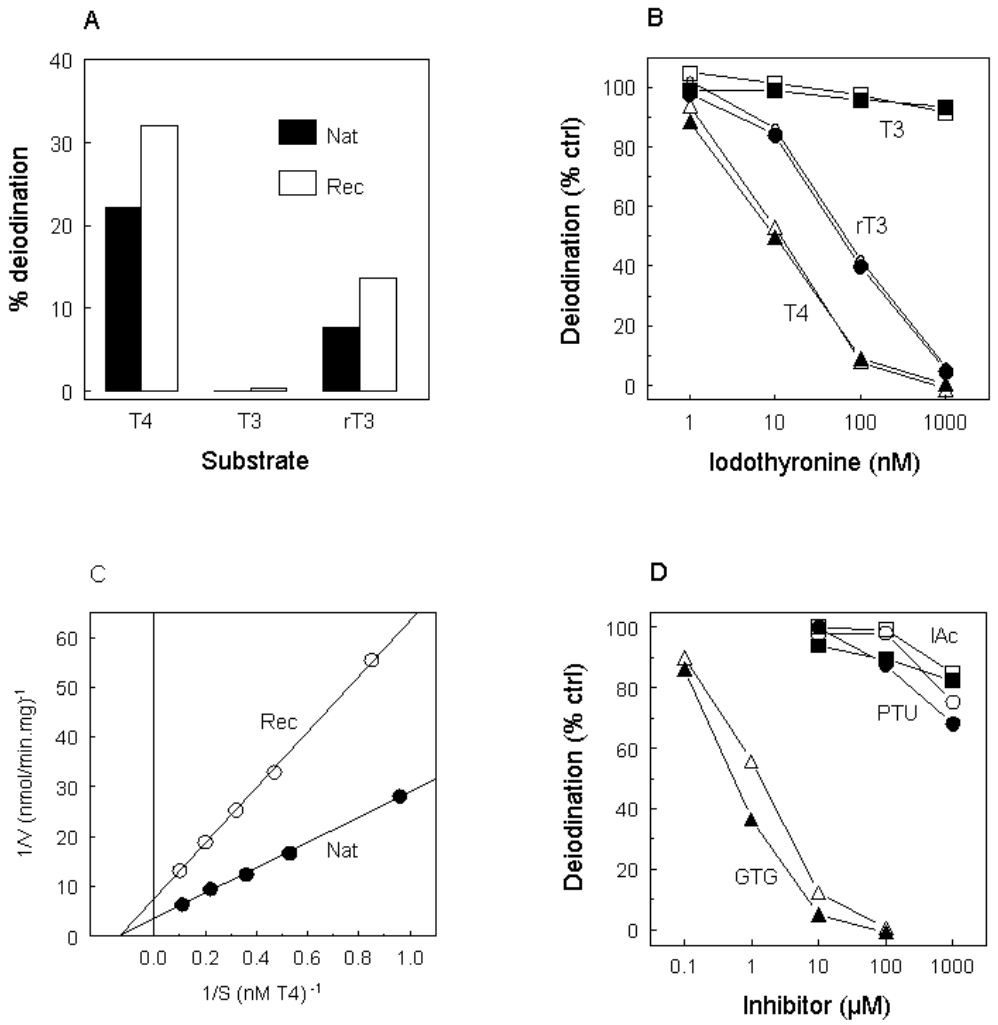


Fig. 4. Characterisation of pig D2 enzyme

A. ORD of T4, T3 or rT3 by recombinant pig D2 (open bars) from transfected COS cell homogenates and native D2 (closed bars) in thyroid homogenate of methimazole treated pig. Assay mixtures contained 1 nM ^{125}I-substrate (100000 cpm) , 25 mM DTT, and 0.2 (lysate) or 0.4 (thyroid homogenate) mg protein/ml and were incubated for 60 min at 37C.

B. Inhibition of the ORD of ^{125}I-T4 by recombinant (open symbols) or native (closed symbols) pig D2 enzyme by unlabeled T4, rT3 and T3. Assay mixtures contained ^{125}I-T4 (100000 cpm), 25 mM DTT, and 0.12 (lysate) or 0.21 (thyroid homogenate) mg protein/ml and were incubated for 60 min at 37 C.

C. Double reciprocal plot of T4 deiodination by recombinant D2 (open symbols) and native D2 enzyme (closed symbols).

D. Inhibition of the ORD of ^{125}I-T4 by recombinant (open symbols) or native D2 (closed symbols) by GTG (0.1 – 100 µM), PTU or IAc (10 – 1000 µM). Assay mixtures contained 1 nM ^{125}I-T4 (100000 cpm), 25 mM DTT, and 0.27 (lysate) or 0.2 (thyroid homogenate) mg protein/ml.

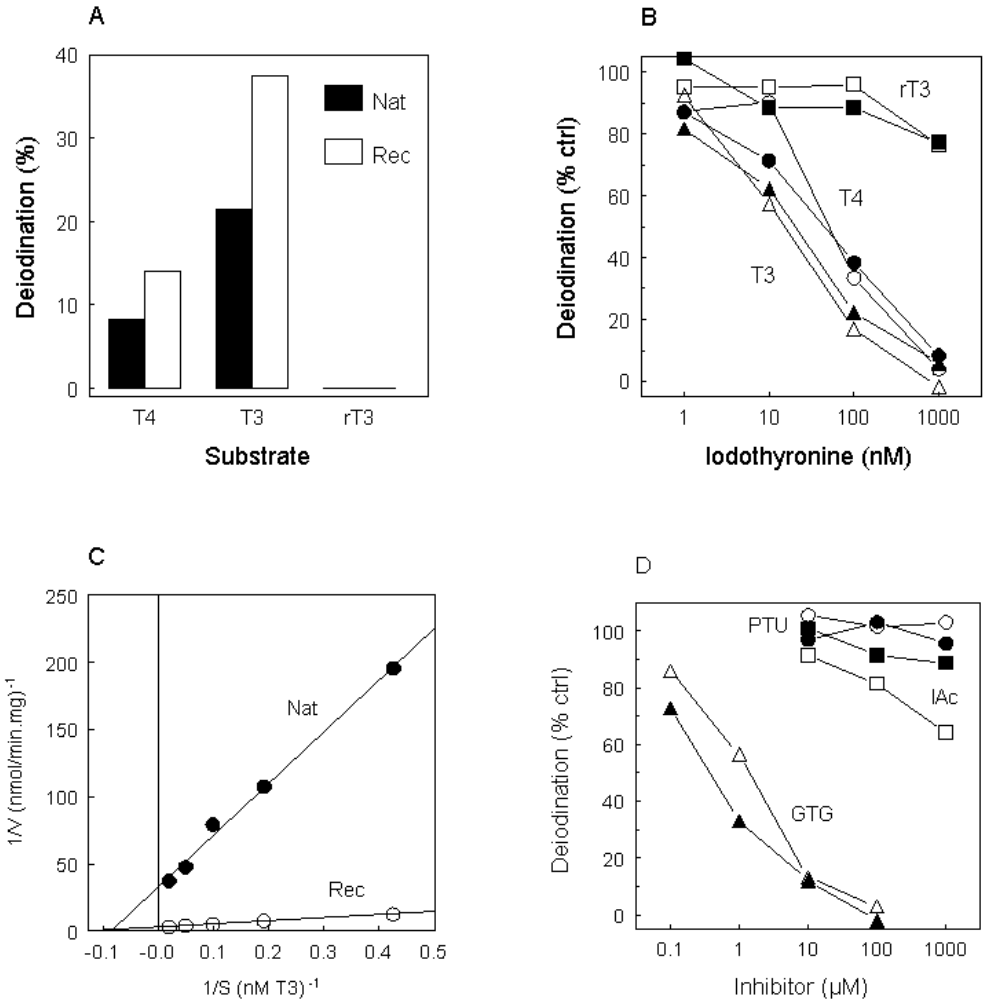


Fig. 5. Characterisation of pig D3 enzyme

A. IRD of T4 and T3 and ORD of rT3 by recombinant pig D3 enzyme (open bars; transfected COS cell homogenates), and native D3 enzyme (closed bars; pig cerebrum microsomal fraction). Assay mixtures contained 10 nM ^{125}I-substrate (200000 cpm), 50 mM DTT and 0.03 (lysate) or 2 (cerebrum microsomes) mg protein/ml and were incubated for 60 min at 37C.

B. Inhibition of the IRD of ^{125}I-T3 by recombinant D3 (open symbols) or native D3 (closed symbols) by increasing concentrations of unlabeled T3, T4 or rT3 (1 – 1000 nM). Conditions were 10 nM ^{125}I-T3 (100000 cpm), 50 mM DTT**,** 0.02 (lysate) or 1 (cerebrum) mg protein/ml.

C. Double reciprocal plot of T3 deiodination by native (closed symbols) and recombinant (open symbols) D3 enzyme.

D. Inhibition of the IRD of ^{125}I-T3 by recombinant (open symbols) or native (closed symbols), by GTG (0.1 – 100 µM), PTU or IAc (10 – 1000 µM). Assay mixtures contained 10 nM ^{125}I-T3, 50 mM DTT, and 1 (cerebrum microsomes) or 0.02 (lysate) mg protein/ml.

Table 3. Apparent K_m values for preferred substrates of porcine iodothyronine deiodinases

Enzyme	Substrate	mM DTT	K_m[a]	
			Native	Recombinant
pD1	rT3	10	0.16 ± 0.04 μM	0.17 ± 0.01 μM
pD2	T4	25	8.7 ± 4.9 nM	8.0 ± 2.0 nM
pD3	T3	50	22 ± 10 nM	15 ± 4 nM

[a]) Mean ± SD of 3-4 experiments. The origin of the native deiodinases was liver (D1), thyroid (D2) and cerebrum (D3).

For comparison with the catalytic activity of recombinant pD2, pig thyroid homogenate (from methimazole-treated pigs) was used as a source of native pD2 (Fig. 4). Native and recombinant pD2 demonstrated 1) ORD activity with T4 and rT3, but no IRD activity with these iodothyronines or T3 as substrates (Fig. 4A); 2) higher rates for ORD of T4 than of rT3 (Fig. 4A); 3) identical dose-dependent inhibition of the ORD of [^{125}I]T4 by unlabeled iodothyronines, with approximate IC_{50} values of 10 nM T4, 100 nM rT3, and »1000 nM T3 (Fig. 4B); 4) identical apparent K_m values for T4 (≈8 nM) (Fig. 4C and Table 3); and 5) similar effects of inhibitors, with approximate IC_{50} values of 1 μM GTG, >1000 μM IAc, and >1000 μM PTU (Fig. 4D). These results demonstrate that native and recombinant pD2 are identical with regard to catalytic activity, substrate specificity, inhibition by GTG, and that these properties are very similar to those previously reported for human and rat D2 (17, 20).

To compare the catalytic activities of native and recombinant pD3, pig cerebrum microsomal preparations were used as a source of native enzyme (Fig. 5). Native and recombinant pD3 showed 1) IRD activity with T4 and T3, but no ORD activity with these iodothyronines or rT3 as substrates (Figure 5A); 2) higher rates for IRD of T3 than of T4 (Fig. 5A); 3) similar dose-dependent inhibition of the IRD of [^{125}I]T3 by unlabeled iodothyronines, with approximate IC_{50} values of 20 nM T3, 50 nM T4 and »1000 nM rT3 (Fig. 5B); 4) identical apparent K_m values for T3 (≈20 nM) (Fig. 5C; Table 3); and 5)

similar sensitivity to inhibitors, with approximate IC_{50} values of 1 μM GTG, >1000 μM IAc, and >1000 μM PTU (Fig. 5D). These results show that native and recombinant pD3 have identical properties regarding catalytic activity, substrate specificity, and inhibitor sensitivity, which are in good agreement with those previously reported for human and rat D3 (33).

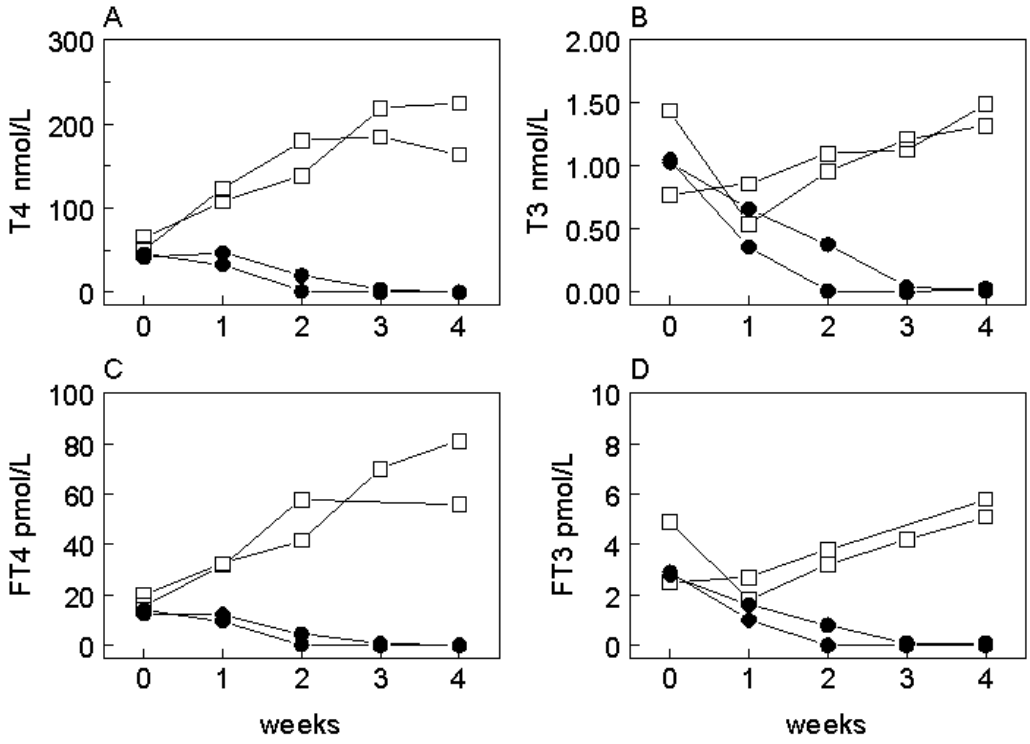


Fig. 6. Plasma T4 and T3 levels (total and free). Pigs were treated with methimazole (closed symbols, n = 2) or T4 (open symbols, n =2) during four weeks as described in *Materials and Methods*. Once every week blood samples were collected and total T4 levels were measured to ensure adequacy of the treatment. At the end of the four week treatment period all plasma samples were measured in the same assay for total as well as free T4 and T3 (see *Materials and Methods*). The total T4 and T3 plasma levels of the untreated pigs were similar to the levels at week 0 of the treated pigs, and did not change over the four week period (not shown).

Table 4 Haemodynamic parameters and body / tissue weight of experimental animals

Parameter	Unit	Hypothyroid	Euthyroid	Hyperthyroid
heart rate	bpm	88 (85, 91)	122 ± 7	135 (127, 142)
mean aortic pressure	mm Hg	81 (77, 85)	91 ± 5	97 (92, 102)
LV systolic pressure	mm Hg	93 (88, 95)	106 ± 4	113 (107, 119)
LV dP/dt_{max}	mm Hg/s	1030(1026,1035)	1815 ± 145	1780(1476,2079)
cardiac output	L /min	2.4 (2.2, 2.6)	2.8 ± 0.2	3.5 (3.0, 4.0)
body weight	kg	36.1 (36.1, 36.1)	37.9 ± 1.7	33.4 (32.1, 34.6)
growth	kg / week	3.4 (3.2, 3.5)	3.1 ± 0.1	2.4 (2.3, 2.4)
thyroid weight	gram	6.7 (6.5, 6.9)	3.1 ± 0.1	2.2 (2.0, 2.4)
thyroid / body wt	mg / kg	186	82	66

For hypothyroid and hyperthyroid pigs (n = 2) the mean value with individual values in brackets are given. For euthyroid pigs (n = 4) the mean ± SEM is given. LV = left ventricle.

Tissue distribution and thyroid state-dependent regulation of pig deiodinases

To study the regulation of pD1-3 in several tissues by thyroid state, 2 pigs were made hypothyroid by methimazole treatment, 2 pigs were made hyperthyroid by high-dose T4 treatment, and 4 untreated pigs were studied as euthyroid controls. Treatment with methimazole resulted in a decrease in serum T4 and T3 levels from about 35 and 0.8 nmol/l, respectively, at baseline to below the detection limit within 2 – 3 weeks in both pigs (Fig. 6). Four weeks after onset of treatment, animals showed a 25 % decrease in heart rate, a 15 % decrease in cardiac output, and a 40 % decrease in $LVdP/dt_{max}$ *versus* euthyroid pigs, whereas body weight gain was normal (Table 4). The thyroid weight, corrected for body weight, doubled in four weeks (Table 4). The porcine TSHβ subunit is highly homologous (80%) with the human TSHβ subunit (51). Nevertheless, due to poor cross reactivity of porcine TSH in our human TSH assay (Immulite 2000, DPC) we could not detect TSH in serum from euthyroid animals, but only in serum from hypothyroid pigs (data not shown). Conversely, treatment with L-thyroxine caused a 4-fold increase of serum T4 levels after 2 – 3 weeks of treatment (Fig. 6). Four weeks after the onset of

treatment, animals showed slight increases in heart rate (15%) and cardiac output (20%) compared to euthyroid pigs, despite a 10% lower body weight (Table 4).

Tissue deiodinase activities were assayed in homogenates and microsomal fractions, except for pituitary where analyses were done only in homogenates. Only deiodinase activities determined in homogenates are presented here (Fig. 7). Activities expressed per mg protein were 2 to 5-fold higher in microsomes than in homogenates, but similar thyroid state–dependent changes were observed in both fractions. In euthyroid pigs, highest D1 activities were observed in liver. Hepatic D1 activities were 20% lower in methimazole-treated pigs than in control animals, but they were markedly (> 3-fold) increased in livers from hyperthyroid pigs (Fig. 7A). Renal D1 activity was not only markedly (> 3-fold) increased in the hyperthyroid animals but also (2-fold) in the methimazole-treated pigs (Fig. 7A). Thyroid and skeletal muscle showed low D1 activity (< 0.1 pmol/min.mg protein), which was not regulated by thyroid state (not shown). No significant D1 activity was detected in brain and pituitary homogenates.

We have recently developed an anti-D1 antiserum, which cross-reacts with cat and porcine D1 protein (47). Microsomal fractions from liver, kidney and thyroid were analyzed by immunoblotting with this antiserum. In microsomal fractions prepared from livers of hyperthyroid pigs the amount of D1 protein was similar or slightly increased compared to microsomal fractions of euthyroid livers (Fig. 8). In microsomal fractions of liver from hypothyroid animals the amount of D1 protein was strongly reduced. In microsomal fractions prepared from kidney (Fig. 8) essentially the same results were obtained, i.e. strong decrease of D1 protein content in preparations from hypothyroid pigs compared to euthyroid and hyperthyroid pigs. In microsomal fractions of thyroid tissue no D1 protein could be detected on Western blots, in line with the very low D1 activities.

BrAc[^{125}I]T3 has been used extensively as affinity label for D1, allowing the specific identification and quantitation of D1 in liver microsomal fractions from many species (12). Affinity labeling was used here for the semi-quantitative analysis of D1 protein in liver and kidney microsomal fractions (Fig. 9). Three prominent radioactively labeled protein bands were visible with apparent molecular masses of 27 kDa, 32 kDa and 56 kDa respectively. The 56 kDa protein was previously identified as protein disulfide isomerase (12). The 32 kDa protein band is not identical to D1 protein since its labeling could not be prevented by

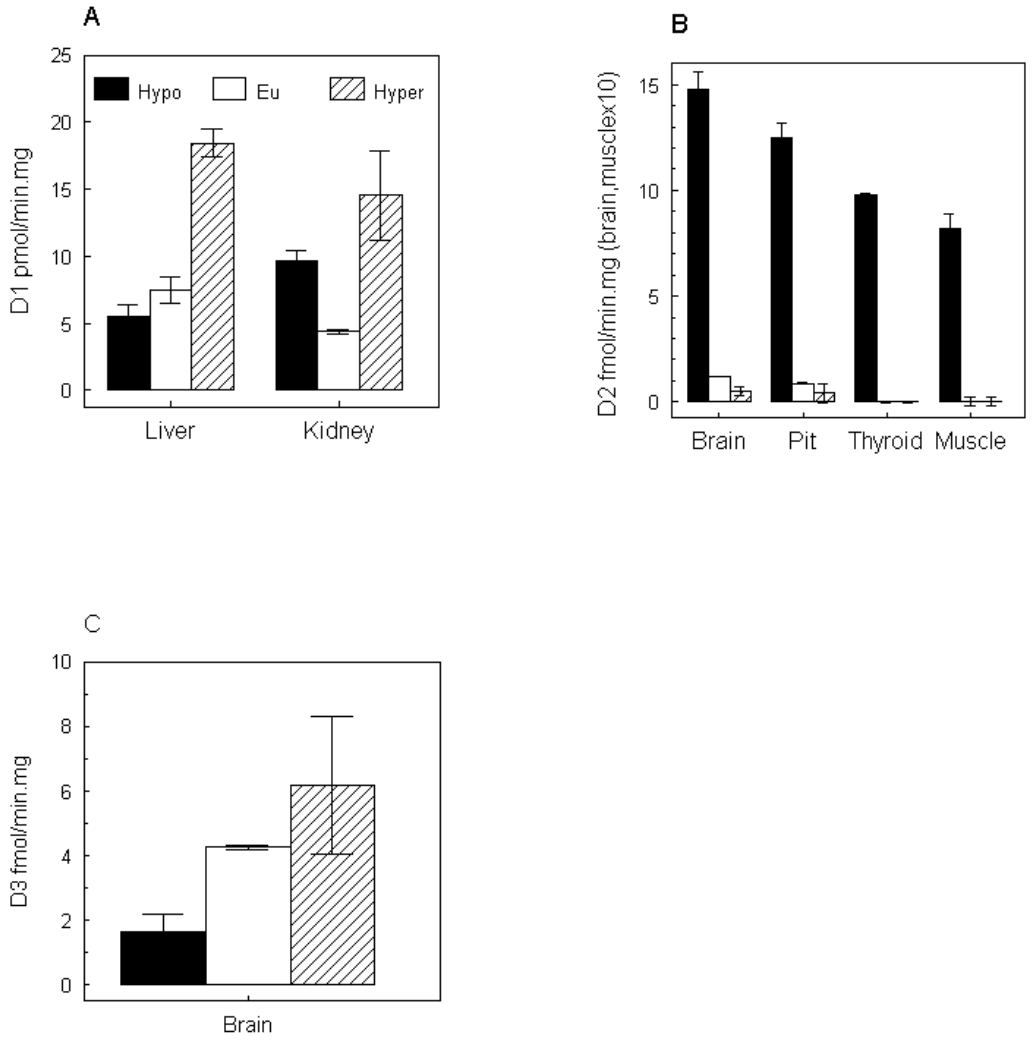
rT3 and PTU (12). The 27 kDa protein band represents D1 protein since affinity labeling could be prevented by rT3 and PTU incubation (12), and it comigrates with the 27 kDa D1 protein present in homogenates from COS cells transfected with the pD1 expression vector (Fig. 9). The labeling intensities of the 27 kDa D1 protein strongly correlate with the D1 activities measured in liver and kidney microsomal fractions from euthyroid, hypothyroid and hyperthyroid pigs. This is especially apparent for kidney microsomal fractions in which the D1 labeling intensities in both the hypothyroid and hyperthyroid state are stronger than in the euthyroid state, in line with the D1 activity levels (Fig. 7A and 9).

In euthyroid pigs, D2 activity was higher in pituitary than in brain (cerebrum), whereas D2 activity was low to undetectable in thyroid and skeletal muscle (Fig. 7B). D2 activities in brain and pituitary were increased >10-fold in hypothyroid pigs and decreased by 40% in hyperthyroid pigs compared with euthyroid controls. A dramatic induction of D2 activity was observed in thyroid of hypothyroid pigs to levels similar to those in hypothyroid pituitary. Furthermore, thyroid weight had increased markedly after methimazole treatment compared with control and T4-treated pigs (Table 4). A striking induction of D2 expression was also seen in skeletal muscle (*Musculus iliopsoas*) to levels approaching those in hypothyroid brain (Fig. 7B). D2 activities were undetectable in liver and kidney even in hypothyroid animals.

Irrespective of thyroid state, significant D3 activities were only detected in brain (cerebrum). Cerebral D3 activity was increased by 40% in hyperthyroid pigs, and decreased by > 50% in hypothyroid pigs *versus* euthyroid controls (Fig. 7C).

Porcine heart homogenates from euthyroid control pigs, hypothyroid pigs and hyperthyroid pigs were analyzed for D2 as well as D3 activity. Hearts were subdivided into left and right ventricle (mainly ventricle wall), left atrium, right atrium and atrium septum. Rather low D2 activities (< 0.1 fmol/min.mg protein) and D3 activities (< 0.1 fmol/min.mg protein) could be detected in these samples, irrespective of thyroid state (not shown).

Fig. 7. Thyroid state-dependent regulation of deiodinase activity in various tissues



A. D1 activities (mean and range shown) in liver and kidney homogenates of methimazole-treated pigs (hypo/closed bars, n = 2), thyroxine-treated pigs (hyper/hatched bars, n = 2) or untreated pigs (eu/open bars, n = 4). Assay mixtures contained 0.1 μM ^{125}I-rT3 (100000 cpm), 10 mM DTT, 0.1 mg protein/ml and were incubated for 30 min at 37C.
B. D2 activities (mean and range shown) in cerebrum, pituitary, thyroid and skeletal muscle (*Musculus iliopsoas*) homogenates of methimazole-treated pigs (closed bars, n = 2), thyroxine treated pigs (hatched bars, n = 2) and untreated pigs (open bars, n = 4). Note that the D2 activities in cerebrum and muscle homogenates are multiplied by a factor 10. Assay mixtures contained 1 nM ^{125}I-T4 (100000 cpm), 25 mM DTT, 1 mg protein/ml and were incubated for 60 min at 37C.
C. D3 activity (mean and range shown) in cerebrum homogenates of methimazole-treated pigs (closed bars, n = 2), thyroxine-treated pigs (hatched bars, n = 2) or untreated pigs (open bars, n = 4). Assay mixtures contained 1 nM ^{125}I-T3 (200000 cpm), 50 mM DTT, 1 mg protein/ml and were incubated for 60 min at 37C.

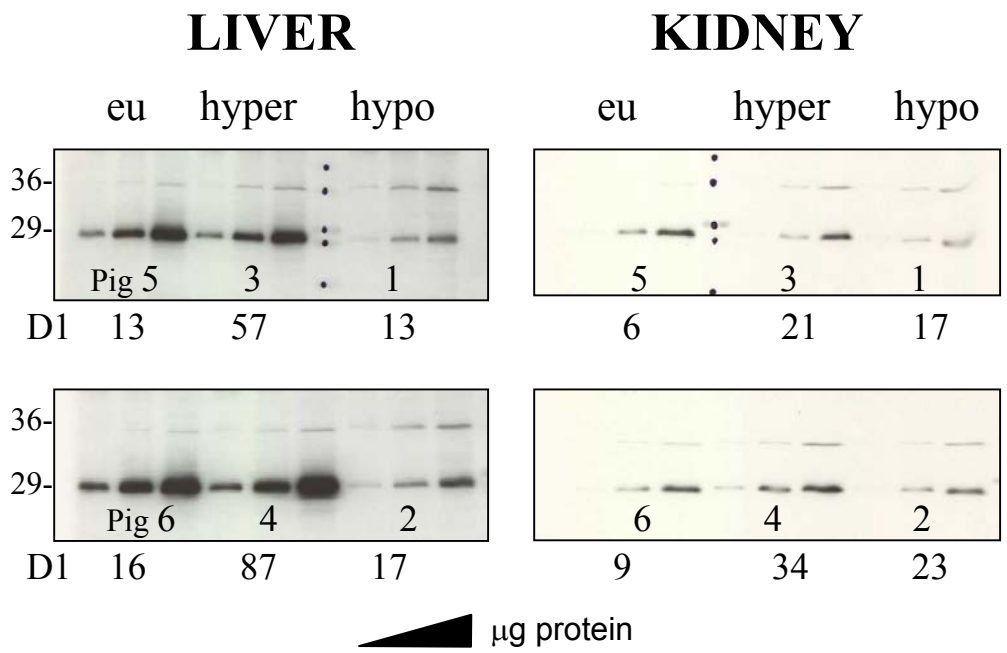


Fig. 8. Western blot analysis of D1 protein (apparent molecular mass 27 kDa) in porcine liver and kidney microsomal fractions. Increasing amounts of microsomal fractions (2.5 – 25 µg protein, supplemented with BSA to 25 µg), were probed with a anti-D1 antiserum generated against an 14-amino acid peptide corresponding to the C-terminal sequence of human D1 *(see Materials and Methods*). Pig number 1 and 2 are methimazole-treated; number 3 and 4 are thyroxine-treated; and number 5 and 6 are untreated. D1 activity values (pmol rT3/min.mg microsomal protein) are indicated. Assay conditions were 0.1 µM ^{125}I-rT3, 10 mM DTT and 0.01 mg microsomal protein/ml. Migration distances of molecular mass markers (kilodaltons) are indicated on the left.

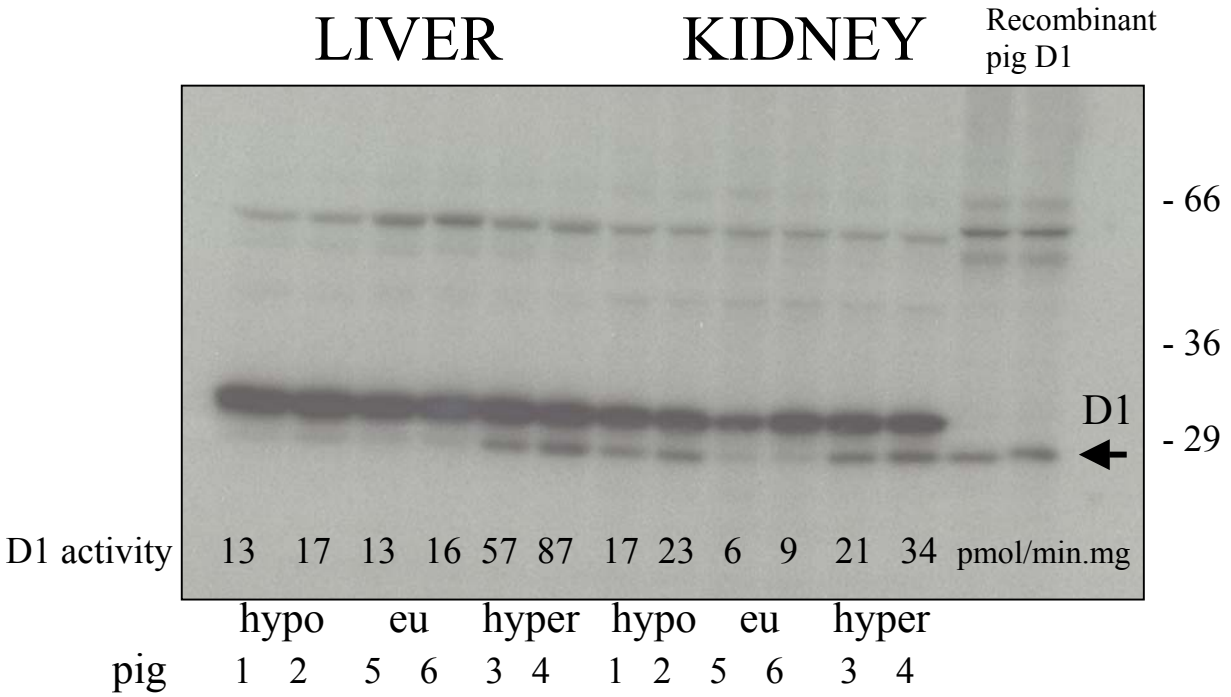


Fig. 9. Labeling patterns obtained by SDS-PAGE and autoradiography after reaction of liver and kidney microsomal fractions (100 µg protein) with BrAc[^{125}I]T3 in the presence of 10 mM DTT at 37 C. Pig number (indicated at bottom) 1 and 2 are methimazole-treated; number 3 and 4 are thyroxine-treated; and number 5 and 6 are untreated pigs. D1 activity values (pmol rT3/min.mg microsomal protein) are indicated inside the autoradiogram. Migration distances of molecular mass markers (kilodaltons) are indicated on the right.

DISCUSSION

Our studies demonstrate a high degree of homology between the amino acid sequences of the porcine iodothyronine deiodinases and the corresponding human enzymes. Also the catalytic properties of the pig deiodinases are virtually identical to those previously reported for the human and rat enzymes. Thus, pD1 possesses both ORD and IRD activities, has apparent K_m values in the micromolar range, accepts rT3 as the preferred (ORD) substrate, and shows markedly facilitated IRD of sulfated *versus* nonsulfated T3. Sulfation has also been reported to facilitate the IRD of T4 by human and rat D1 (1-3, 8), but this was not studied here with pD1. The only difference noted between pig, human and rat D1 was the higher susceptibility of 3,3'-T2S to undergo IRD by pD1 than by human D1 and, in particular, rat D1. Much like the enzyme in human and rats, pD1 is extremely sensitive to inhibition by GTG, IAc, and PTU.

The catalytic properties of pD2 and pD3 are also identical to those reported for the corresponding human and rat deiodinases. Thus, pD2 only shows ORD activity, prefers T4 to rT3 as the substrate, and shows apparent K_m values in the nanomolar range. Pig D3 has only IRD activity, prefers T3 to T4 as the substrate, and shows apparent K_m values intermediate between those of pD1 and pD2. D2 and D3 from humans and rats do not catalyze the deiodination of sulfated substrates, but this was not studied here with pD2 and pD3. Much like the human and rat enzymes, pD2 and pD3 are only inhibited by GTG at 100-fold higher concentrations than those required for pD1 inhibition, whereas pD2 and pD3 are hardly affected by IAc and PTU. GTG and IAc are thought to inhibit D1 by reacting with the selenolate (Se^-) form of the catalytic SeC residue, whereas PTU is thought to react with the selenenyl iodide (SeI) intermediate generated during catalysis. The much lower potencies of the effects of these inhibitors on D2 and D3 suggest that these enzymes follow a catalytic mechanism different from that of D1, although they also contain a SeC residue in their active centers (45-46).

To further investigate the suitability of the pig as an animal model for human thyroid hormone metabolism we studied the tissue distribution of the different deiodinases as well as their regulation by thyroid state. Treatment with methimazole successfully induced hypothyroidism as indicated by the reduction of both plasma T4 and T3 (both total and

free) to undetectable levels, the large increase in thyroid weight, and the marked decrease in cardiac output. Conversely, treatment with T4 resulted in large increases in serum total and free T4, decreases in thyroid and body weight, and increases in heart rate and cardiac output, attesting to the hyperthyroid state of the animals. This was supported by the significant increases in D1 activities in liver and kidney of the T4-treated pigs. Previous studies have shown increased D1 activity in liver homogenates of hyperthyroid rats (52). In addition, a close correlation between rodent hepatic and renal D1 mRNA levels and enzyme activity was found (53-55), suggesting predominant regulation at the pretranslational level. In studies with thyroid hormone receptor (TR) deficient mice it was established that TRβ is mainly responsible for D1 regulation in liver, while in kidney regulation relied solely on TRβ (56). Identification of T3 receptor-binding response elements (TREs) in the human D1 gene promoter (15, 57) further indicates that the thyroid hormone regulation of D1 is exerted at the level of gene transcription. Whether similar TREs are present in the porcine D1 promoter remains to be investigated.

Remarkably, D1 activity showed only a small decrease in liver and actually a marked increase in kidney of hypothyroid pigs. The latter, that is the increased activity in kidney of hypothyroid pigs in comparison to euthyroid pigs was reflected in increased labeling intensity upon affinity labeling with BrAcT3. In general the BrAcT3 affinity labeling data correlates strongly with the D1 activity measurements. Meanwhile, the immunoblotting experiments revealed decreased D1 protein content in livers and kidneys of hypothyroid animals, while the D1 protein content of tissue from euthyroid and hyperthyroid pigs was very similar. There appears to exist a discrepancy between the D1 protein content as determined by immunoblotting and the D1 activity measurements. This is the case for the hypothyroid pigs, in which activities are similar (liver) or increased (kidney) while the D1 protein content is decreased (liver and kidney), compared to euthyroid animals. Also, for hyperthyroid pigs the activity is clearly increased in liver and kidney compared to euthyroid pigs, while the protein content is similar for euthyroid and hyperthyroid pigs. We have recently observed alternative splicing of D1 mRNA in human liver (58). Nine D1 mRNA variants were identified sofar, all encoding truncated proteins from which two still contain the catalytic center (see GenBank accession numbers AY560374 – AY560383). The epitope of our D1 antiserum is at the C-terminus, and it would not detect all of these

D1 variants provided that these also exist at the protein level. So, it could be that in porcine liver and kidney variant D1 proteins exist that contribute to D1 activity, which is not detected by immunoblotting. Efforts to raise polyclonal D1 antisera directed against different epitope(s) have not been successful sofar.

To our knowledge this is the first study that compares liver and kidney D1 protein content determined by immunoblotting and activity measurements in euthyroid, hypothyroid, and hyperthyroid animals. Only one previous study by DePalo et al (59) showed increased abundance of the 27 kDa D1 protein band on Western blots of liver microsomes from hyperthyroid rats as compared to samples from hypothyroid rats, which is in line with our results. In that study no immunoblot data for euthyroid rats was presented and also no D1 activity measurements. In a human hepatoma cell line (HepG2) it was found that T3 treatment influences the alternative splicing of TRα mRNA, thus changing the balance towards TRα2 encoding mRNA (60). Although regulation of D1 expression by T3 is generally considered to occur mainly at the transcriptional level (15, 53-57), effects of T3 on D1 splicing cannot be excluded, and remain to be investigated.

Finally, we cannot exclude that the lack of a large reduction in hepatic D1 activity and the increase in renal D1 activity in methimazole-treated rats could represent direct effects of methimazole rather than the hypothyroid state it induces. This has been demonstrated for the marked induction of phenol UDP-glucuronyltransferase activity in livers of methimazole-treated rats, which was not prevented by administration of T4 replacement doses (61).

The regulation of D2 expression by thyroid state has been extensively studied in rat brain and pituitary (17, 24-26, 62-64). Our findings regarding the effects of hypo- and hyperthyroidism on D2 activity in pig brain and pituitary are in excellent agreement with these previous studies in rats. Thus, brain and pituitary D2 activities were strongly increased in hypothyroid pigs and markedly decreased in hyperthyroid animals compared with the euthyroid controls. As demonstrated in rats, the negative control of D2 expression in brain and pituitary by thyroid hormone involves two different mechanisms (62). Firstly, down-regulation of D2 mRNA expression by thyroid hormone is probably mediated by the nuclear T3 receptor, although a putative negative TRE in the promoter region of the D2 gene remains to be identified (39, 65). More importantly, D2 also undergoes substrate-

induced enzyme inactivation by selective proteolysis, which is exerted by the substrates T4 and rT3 rather than by T3 (26, 28, 64, 67).

One of the remarkable findings in our study is the expression of high D2 activity in thyroid of hypothyroid pigs, while little thyroidal D2 activity was found in euthyroid animals. Normal human thyroid tissue expresses D2, thus contributing to the plasma T3 pool (18, 21). In patients with Graves' disease or hyperfunctioning adenomas D2 activities increase significantly, sometimes causing relatively high circulating free T3 levels (18, 21, 68). Thyroid function is normally undisturbed in patients with thyroid carcinoma, but in some patients with large or widely metastatic follicular carcinoma increased T4 to T3 conversion due to overexpression of D2 was found (69). *In vitro* studies using human thyroid cells have shown that D2 expression is up-regulated by TSH through the cAMP-protein kinase A pathway (18, 21, 38). The effect of cAMP is exerted at the pretranslational level, probably by stimulation of D2 gene transcription as suggested by the identification of a cAMP response element (CRE) in the promoter region of the human D2 gene (66, 70). The expression of D2 in human but not in rat thyroid has been associated with the presence of a thyroid transcription factor 1 (TTF1) response element in the D2 gene promoter in humans but not in rats (39). It is very likely that such cAMP and TTF1 response elements are also present in the promoter of the pig D2 gene, and that the increased D2 activity in hypothyroid pigs is the consequence of elevated TSH levels acting through the cAMP-protein kinase A pathway.

Perhaps the most striking finding in our study is the expression of high D2 activity in skeletal muscle of hypothyroid pigs in contrast to the insignificant D2 activity in skeletal muscle of euthyroid animals. Expression of D2 mRNA and activity in human skeletal muscle has been reported (17, 20). Also, cultured human skeletal muscle cells express D2 mRNA and activity in particular in the absence of thyroid hormone and after β-adrenergic stimulation of the cAMP-mediated pathway (19). D2 expression in skeletal muscle may play a role in local T3 production in particular in hypothyroid subjects. The increased D2 activity in skeletal muscle of hypothyroid pigs might in part be explained by a reduction of T4-induced D2 proteolysis (67).

Significant expression of D3 was only observed in brain (cerebrum) but not in other tissues. The positive regulation of brain D3 expression by thyroid state is in agreement

with previous studies of the regulation of D3 in rat brain (34-35). The mechanism of this thyroid hormone-dependent regulation of D3 expression remains to be established.

We could detect rather low D2 and D3 enzyme activities in porcine heart samples, which were not regulated by thyroid state. Several studies have reported D2 and D3 activity in heart homogenates, albeit at low levels. In rat heart D3 activity was induced during hypertrophy and cardiac failure (71). Prolonged treatment with methimazole increased D2 activity in mouse and rat heart (72). In several studies D2 mRNA was detected in rodent or human heart, either by RT-PCR (72, 73) or by Northern blotting (20). At the moment no studies are available which describe D2 or D3 activities in human heart samples. It is obvious that more investigations are needed, in particular with regard to possible changes in deiodinase activities in human or porcine heart during ventricular hypertrophy, cardiac failure and after myocardial infarction.

In conclusion, the pig appears to be a good animal model for human thyroid hormone metabolism, considering the high degree of homology between the structures, functional properties, tissue distribution, and thyroid-state dependent regulation of the porcine and human iodothyronine deiodinases. The expression of D2 in porcine thyroid and skeletal muscle is of particular interest for studies on the importance of the enzyme in these tissues of (hypothyroid) human subjects.

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CHAPTER 4

TYPE I IODOTHYRONINE DEIODINASE SPLICE VARIANTS IN HUMAN TISSUES

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ABSTRACT

The gene for human type I deiodinase (D1) is located on chromosome 1p32-p33 and consists of 4 exons. Exon 1 codes for the 5' untranslated region (UTR) and amino acids (AAs) 1-112; exon 2 for AAs 113-160, including selenocysteine (Sec) in position 126; exon 3 for AAs 161-227; and exon IV for AAs 228-249 and the 3' UTR. We have studied the possible alternative splicing of D1 mRNA in human tissues. Total RNA was isolated from human liver, kidney, and thyroid, and a reverse transcription-polymerase chain reaction (RT-PCR) was performed using primers located over the translation start and stop codons. Agarose gel electrophoresis showed multiple bands ranging from ~400-750 kb. The PCR products were cloned and 60 clones were sequenced. Ten D1 variants were identified, with 24 clones for variant a, 9 for variant e, 5 for variant f, 4 for variant l, 2 for variant b, and 1 for each variant c, d, k, m and s. hD1a represents wild-type D1; all other variants code for shorter proteins. In these splice variants, part of exon 1 (b,c,f,k,m,s), all of exon 2 (d,l,s), and/or all of exon 3 (c,e,l) are deleted. Genescan analysis confirmed the presence of multiple D1 mRNA splice variants in human tissues. In addition to wild-type hD1, only variant proteins b and e have the catalytic Sec residue, and only variants b and d have the C-terminal epitope used for generation of hD1-specific antibodies. However, immunoblots of human liver, kidney and thyroid only show the presence of wild-type protein, suggesting insignificant expression of the splice variants at the protein level. The function of hD1 mRNA splice variation remains to be determined.

INTRODUCTION

Thyroid hormone plays a major role in many biological processes including development, growth and basal metabolism (1-3) . It is produced by the thyroid as the biologically inactive precursor T4 and has to be converted to the biologically active hormone T3 (4). T4 is thought to be inactive because of its low affinity for the nuclear T3 receptor (2, 5). Outer ring deiodination (ORD) of T4 to T3 is catalyzed by both type I (D1) and type II (D2) iodothyronine deiodinases (3). In mammals, D1 is expressed at high levels in liver and kidney, and in some species also in thyroid. Liver D1 plays a major role in serum T3 production (6, 7). D2 activity is found in anterior pituitary, brain, rat brown adipose tissue, skin, human thyroid gland, and skeletal muscle, and plays a key role in local production of T3 in these tissues (2, 8-10). Whereas D1 and D2 are activating enzymes, D3 inactivates thyroid hormone by inner ring deiodination (IRD), converting T4 to rT3 and T3 to 3,3'-T2. D3 activity is expressed in particular in brain, skin, placenta, pregnant uterus, and different fetal tissues (2, 11-15).

Iodothyronine deiodinases represent a group of homologous selenoenzymes, i.e. selenocysteine (Sec)-containing enzymes, that need thiols as cofactor (3). *In vitro*, DTT serves as thiol donor. Despite its role in serum T3 production, the preferred substrate for D1 is rT3, with the exception of D1 from cat and dog (16-19). The IRD of T4 and T3 by D1 is greatly facilitated by sulfation of these substrates (20-22). Typically, apparent Km values of substrates for D1 are in the μM range, and the enzyme is sensitive to inhibition by propylthiouracil, with the exception of D1 from fish (23). In contrast, T4 is the preferred substrate for D2 and T3 for D3, with apparent Km values in the nM range (3).

The human *Dio1* gene is located on chromosome 1p32-33 and is 17.5 kb in length (24). It contains three introns of 10.1, 1.3 and 3.9 kb, respectively. The mature mRNA is 2.2 kb long, with a coding sequence of 750 bp (25). The catalytic Sec residue in the middle of the human D1 amino acid sequence is encoded by an UGA opal stop codon. Termination of translation normally signaled by this triplet is suppressed in the presence of a so-called Sec insertion sequence (SECIS) in the 3'UTR of the mRNA (3).

Although estimates of the number of protein-encoding human genes vary widely from 28,000 (26) to 120,000 (27) it is clear that through alternative splicing considerably more diversity can be generated at the mRNA level. Previous studies based on comparisons using ESTs, mRNAs, or genomic sequences have estimated that 22-59% of human genes undergo

alternative splicing (28-34). Furthermore, since the number of human expressed mRNA forms is much higher than the number of genes, there is good evidence that alternative splicing plays a very significant role in the complexity of genomic function (33). Whereas the *Dio1* gene consists of 4 exons, *Dio2* has only 2 exons, and *Dio3* is a single-exon gene. Alternative splicing has been described for D2 mRNA (35, 36), but no study has been reporter on possible splice variation in D1. We have studied alternative splicing of human D1 mRNA as a potential mechanism for the regulation of this enzyme. Our approach has been based on the use of RT-PCR to coamplify multiple alternatively spliced mRNA transcripts from the *DIO1* gene followed by cloning as a simple screen to identify variant isoforms as described earlier (37).

MATERIALS AND METHODS

Materials

TRIzol reagent was obtained from GIBCO BRL (Breda, The Netherlands). Oligo(dT), random hexamer primers, dNTP, RNAse inhibitor, 5' first strand buffer, M-MLV reverse transcriptase, agarose and pGEM-T(easy) were from Promega (Madison, WI); recombinant Taq DNA polymerase (5 U/µl) was from Pharmacia Biotech (Roosendaal, The Netherlands); and Qiaprep Spin Miniprep Kit from Qiagen (Hilden, Germany). Synthetic oligonucleotides were ordered from Invitrogen-Life Technologies (Paisley, UK), and FAM-labeled antisense primer was purchased from Biosource Europe S.A. (Nivelles, Belgium). All other reagents were of the highest purity commercially available.

Adult and fetal human liver, adult thyroid and adult kidney samples were obtained as previously described (15, 38, 39), and immediately frozen in liquid nitrogen and stored at –80 C until further analysis. Approval was obtained from the Medical Ethical Committees of the Erasmus University Medical Center, and the University of Dundee Medical School and Ninewells Hospital.

Amplification of the hD1 mRNA coding region

Total RNA was isolated from the different tissue samples (approx. 200 mg) using TRIzol reagent. cDNA was synthesized from 5 µg of total RNA using random hexamer primers as well as oligo(dT) primers and M-MLV reverse transcriptase in a total volume of 50 µl. PCR mixtures contained 2 µl cDNA mixture, 0.8 mM dNTPs, 1.5 mM $MgCl_2$, 0.2

pmol of each primer, and 2 units of recombinant Taq DNA polymerase in 200 mM Tris-HCl (pH 8.4), 500 mM KCl (final volume 50µl). Using genomic information available at the NCBI website (www.ncbi.nlm.nih.gov/LocusLink/LocRpt.cgi?l=1733), flanking primers in the 5' and 3' untranslated regions (UTRs) were designed to detect splice variation in the open reading Thus, 5'-TCTGGCTTTGCCGAGATG-3' (start codon underlined) was used as the forward primer, and 5'- GAGGTATCTGTCCAGATTAAC-3' as the reverse primer (stop codon underlined). PCR was carried out in a Gene AMP PCR 9700 system (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) using the following protocol: initial denaturation for 5 min at 94 C, followed by 35 cycles of 1 min at 94 C, 1 min at 45 C, and 2 min at 72 C, with a final elongation for 7 min at 72 C. PCR products were analyzed on ethidium bromide-stained agarose gels.

Genescan analysis

hD1 mRNA splice variants were also investigated by Genescan analysis. For this purpose, hD1 mRNA coding sequence was amplified by RT-PCR as described above using reverse primer labeled with 6-carboxyfluorescein (FAM) fluorescent dye. PCR fragments were analyzed using an ABI Prism 3100 Genetic Analyzer with Genescan analysis software 1.2, and R0X 2500 as internal size standards (Applied Biosystems).

Sequence analysis

PCR products were TA cloned into the pGEM-T(easy) vector. Plasmids were purified using the Qiaprep Spin Miniprep Kit, and inserts were sequenced (ABI PRISM BigDye Terminator Cycle Sequencing, Applied Biosystems) in both directions using T7 and Sp6 primers. The sequences obtained were aligned with the wild-type (wt) hD1 cDNA sequence (accession code NM_000792) using the Blast program available on the NCBI website.

Immunoblotting of tissue hD1 protein

Human tissue homogenates and microsomal fractions thereof were obtained as previously described (15, 38), and samples containing 20-40 µg protein were separated on 12% SDS-PAGE gels in the Mini-Protean III cel (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. After electrophoresis, proteins were blotted to nitrocellulose membranes, and probed with rabbit polyclonal anti-D1 antiserum 1068 (1:500) as described previously (40). The epitope of this antiserum is located in the C terminus

(amino acids 236-249) of the hD1 protein (16). The intensity of the D1 protein bands was analyzed by densitometry. In control experiments, it was shown that the antiserum does not detect human D2 or D3 protein.

RESULTS

Total RNA was isolated from human tissue samples, *i.e.* 6 adult livers, 13 fetal livers (13 to 33 weeks of gestational age) (not shown), 1 adult thyroid, and 1 adult kidney. RT-PCR was performed as described in the Materials and Methods section using coding sequence-flanking primers. Using these primers, wt hD1 mRNA is expected to produce a PCR fragment of 778 bp. However, agarose gel electrophoresis consistently demonstrated multiple ethidium bromide-stained bands with sizes varying roughly between 400 and 750 bp, the largest band being the most abundant (Fig.1). The patterns observed appeared independent of tissue type and age of the donor.

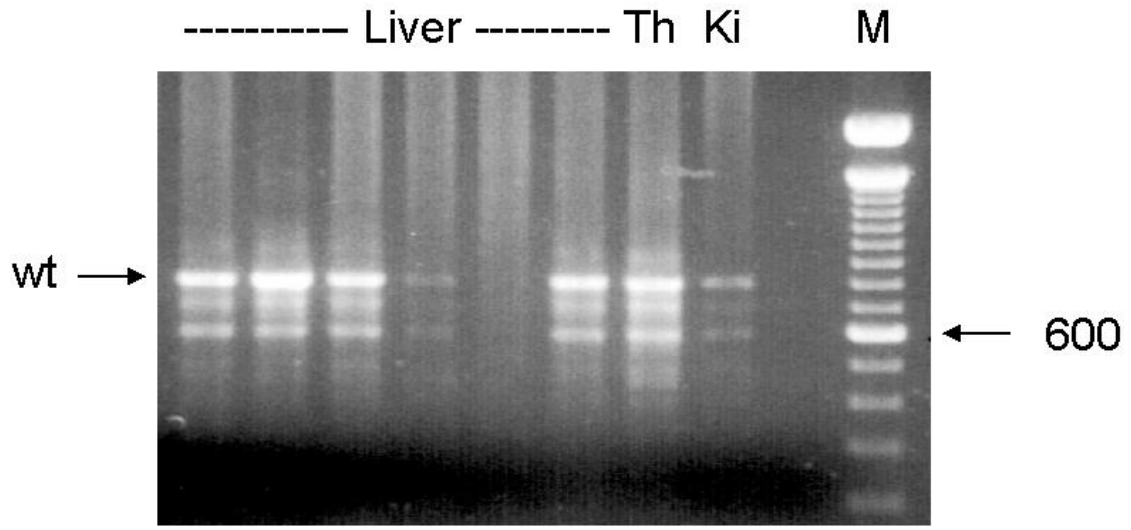

Fig. 1. Data from RT-PCR on RNA isolated from adult liver, thyroid (Th) and kidney (Ki) are shown. The calculated size of hD1 mRNA amounts 778 Bp, indicated by the arrow on the left. Agarose gel electrophoresis demonstrates multiple ethidium bromide-stained bands with sizes varying roughly between 400 and 750 bp. On the right a 100 Bp Marker (M) is shown

To identify the differently sized cDNA fragments obtained, PCR products were AT-cloned, and 8-10 cDNA clones per tissue sample were isolated and sequenced in both directions. In all, 60 clones were obtained and the nucleotide sequences of 49 of those were determined unambiguously. These sequences were compared with the published wt hD1 cDNA sequence using the BLAST program (NCBI website), resulting in the identification of 17 variants, 10 of which appeared to be genuine splice variants.

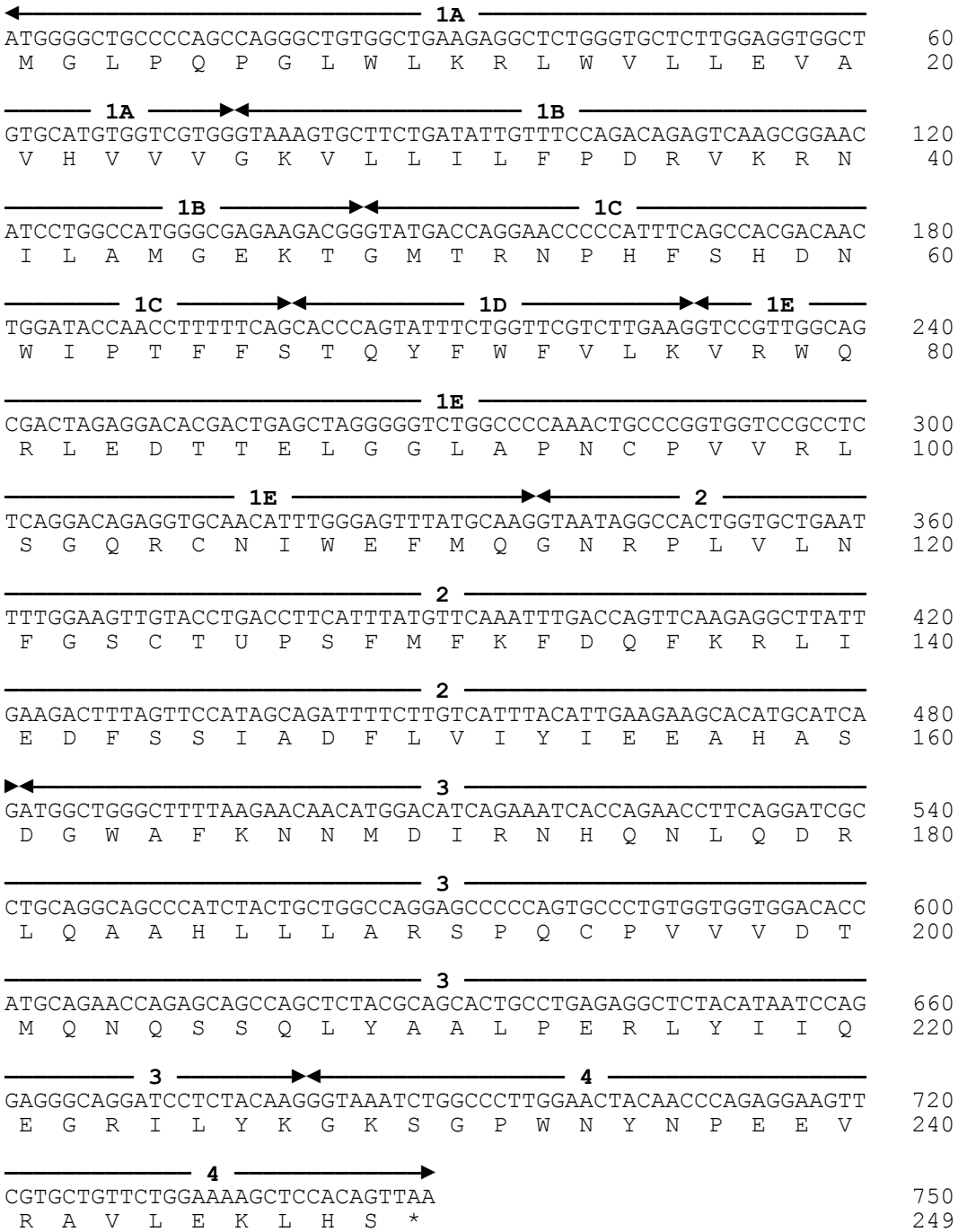

Fig. 2. Coding nucleotide sequence and amino acid sequence of hD1. Exons 1-4 are indicated. Exon 1 is divided in 5 parts (ABCDE) by 4 internal splice sites

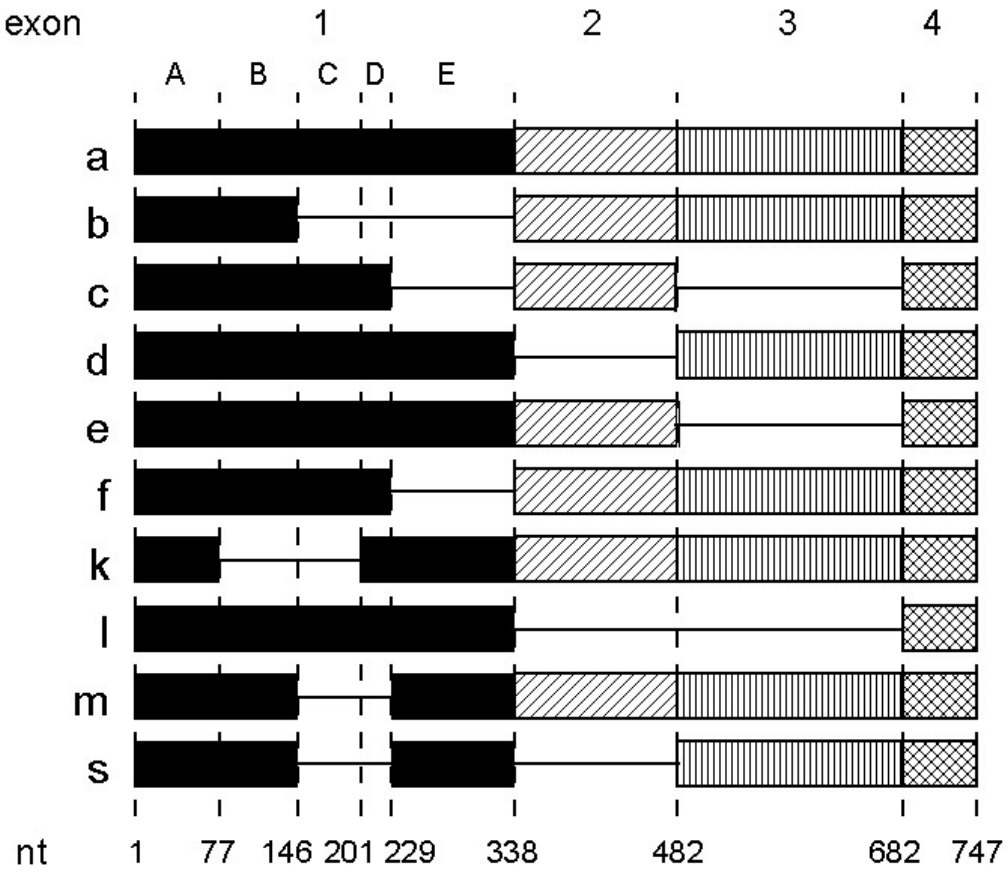

Fig. 3. Identity of the 10 relevant hD1 variants, indicated by the letters shown on the left side of the picture. The 4 exons of hD1 are depicted at the top of the figure and the nucleotides at the bottom.

```
a,d,e,l   ATGGGGCTGCCCCAGCCAGGGCTGTGGCTGAAGAGGCTCTGGGTGCTCTTGGAGGTGGCT   60
b         ATGGGGCTGCCCCAGCCAGGGCTGTGGCTGAAGAGGCTCTGGGTGCTCTTGGAGGTGGCT   60
c,f       ATGGGGCTGCCCCAGCCAGGGCTGTGGCTGAAGAGGCTCTGGGTGCTCTTGGAGGTGGCT   60
k         ATGGGGCTGCCCCAGCCAGGGCTGTGGCTGAAGAGGCTCTGGGTGCTCTTGGAGGTGGCT   60
m,s       ATGGGGCTGCCCCAGCCAGGGCTGTGGCTGAAGAGGCTCTGGGTGCTCTTGGAGGTGGCT   60

a,d,e,l   GTGCATGTGGTCGTGGGTAAAGTGCTTCTGATATTGTTTCCAGACAGAGTCAAGCGGAAC  120
b         GTGCATGTGGTCGTGGGTAAAGTGCTTCTGATATTGTTTCCAGACAGAGTCAAGCGGAAC  120
c,f       GTGCATGTGGTCGTGGGTAAAGTGCTTCTGATATTGTTTCCAGACAGAGTCAAGCGGAAC  120
k         GTGCATGTGGTCGTGG----------------------------------------------   76
m,s       GTGCATGTGGTCGTGGGTAAAGTGCTTCTGATATTGTTTCCAGACAGAGTCAAGCGGAAC  120

a,d,e,l   ATCCTGGCCATGGGCGAGAAGACGGGTATGACCAGGAACCCCCATTTCAGCCACGACAAC  180
b         ATCCTGGCCATGGGCGAGAAGACGG-----------------------------------  145
c,f       ATCCTGGCCATGGGCGAGAAGACGGGTATGACCAGGAACCCCCATTTCAGCCACGACAAC  180
k         ------------------------------------------------------------   76
m,s       ATCCTGGCCATGGGCGAGAAGACGG-----------------------------------  145

a,d,e,l   TGGATACCAACCTTTTTCAGCACCCAGTATTTCTGGTTCGTCTTGAAGGTCCGTTGGCAG  240
b         ------------------------------------------------------------  145
c,f       TGGATACCAACCTTTTTCAGCACCCAGTATTTCTGGTTCGTCTTGAAG------------  228
k         --------------------CACCCAGTATTTCTGGTTCGTCTTGAAGGTCCGTTGGCAG  116
m,s       -------------------------------------------------GTCCGTTGGCAG  157

a,d,e,l   CGACTAGAGGACACGACTGAGCTAGGGGGTCTGGCCCCAAACTGCCCGGTGGTCCGCCTC  300
b         ------------------------------------------------------------  145
c,f       ------------------------------------------------------------  228
k         CGACTAGAGGACACGACTGAGCTAGGGGGTCTGGCCCCAAACTGCCCGGTGGTCCGCCTC  176
m,s       CGACTAGAGGACACGACTGAGCTAGGGGGTCTGGCCCCAAACTGCCCGGTGGTCCGCCTC  217

a,d,e,l   TCAGGACAGAGGTGCAACATTTGGGAGTTTATGCAAG                        337
b         -----------------------------------                        145
c,f       -----------------------------------                        228
k         TCAGGACAGAGGTGCAACATTTGGGAGTTTATGCAAG                        213
m,s       TCAGGACAGAGGTGCAACATTTGGGAGTTTATGCAAG                        254
```

Fig. 4. The nucleotide sequences of exon I of human D1 variants

The identity of these splice variants is explained in Figs. 3 and 4, and the amino acid sequences of relevant variants are given in Fig. 5. They are generated by splicing out of various parts of exon 1, entire exon 2 and/or entire exon 3. In this context, it is important to note that the coding sequence of exon 1 is divided in 5 parts (ABCDE) by 4 internal splice sites, *i.e.* donor GGGT splice sites at the AB (nts 75-78) and BC (nts 144-147) boundaries, an acceptor AGCA splice site at the CD boundary (nts 199-202), and an AGGT donor/acceptor splice site at the DE boundary (nts 227-230).

Variant a shows an intact cDNA sequence, representing wt hD1 mRNA which codes for a protein of 249 amino acids. Although variant b lacks a major part of exon 1 (CDE), the reading frame is maintained, predicting the generation of a protein lacking amino acids 48-112 but with an intact catalytic center and C-terminal half. Exon 1E is deleted from both variants c and f, and in addition variant c also lacks exon 3. The deletion of exon 1E results in a frame shift with early termination of translation, and both variants c and f code for a severely truncated protein lacking the catalytic Sec residue. Exon 2 is deleted from variant d, exon 3 is deleted from variant e, and both exons are deleted from variant l. Although variant d codes for a protein lacking 48 amino acids, including the catalytic Sec residue, the reading frame is maintained and both N and C-terminal regions are intact. In addition to variants a (wt) and b, hD1e is the only other splice variant that still codes for a protein with an intact catalytic center. However, the protein is severely truncated, lacking the C-terminal one-third of the wt deiodinase. Exon 1BC is deleted from variant k, and exon 1CD is deleted from variants m and s, while the latter also lacks the entire exon 2. All these deletions predict major losses of protein structure and function.

Regarding the abundance of the completely characterized splice variants, 49% (24/49) represented variant a (wt D1), 18% (9/49) variant e, 10% (5/49) variant f, 8% (4/49) variant l, and 4% (2/49) variant b, while one cDNA clone (2%) was identified for each variant c, d, k, m and s (Table 1). Genescan analysis of the splice variants was done by RT-PCR of human tissue RNA under the same conditions except for the use of a FAM-labeled downstream primer. This provided results essentially in agreement with our cloning experiments, indicating particularly high abundance of variants e and f in addition to wt hD1 messenger (not shown). Supporting the relatively high abundance of variant e, screening of GenBank revealed 2 human expressed sequence tags (ESTs) and 1 rat EST with the corresponding nucleotide sequence (Table 1). In addition, cDNA sequences corresponding to variants b, d and f have been identified in GenBank which were obtained by analysis of a single gene

library of human liver D1, using a similar method as we used.

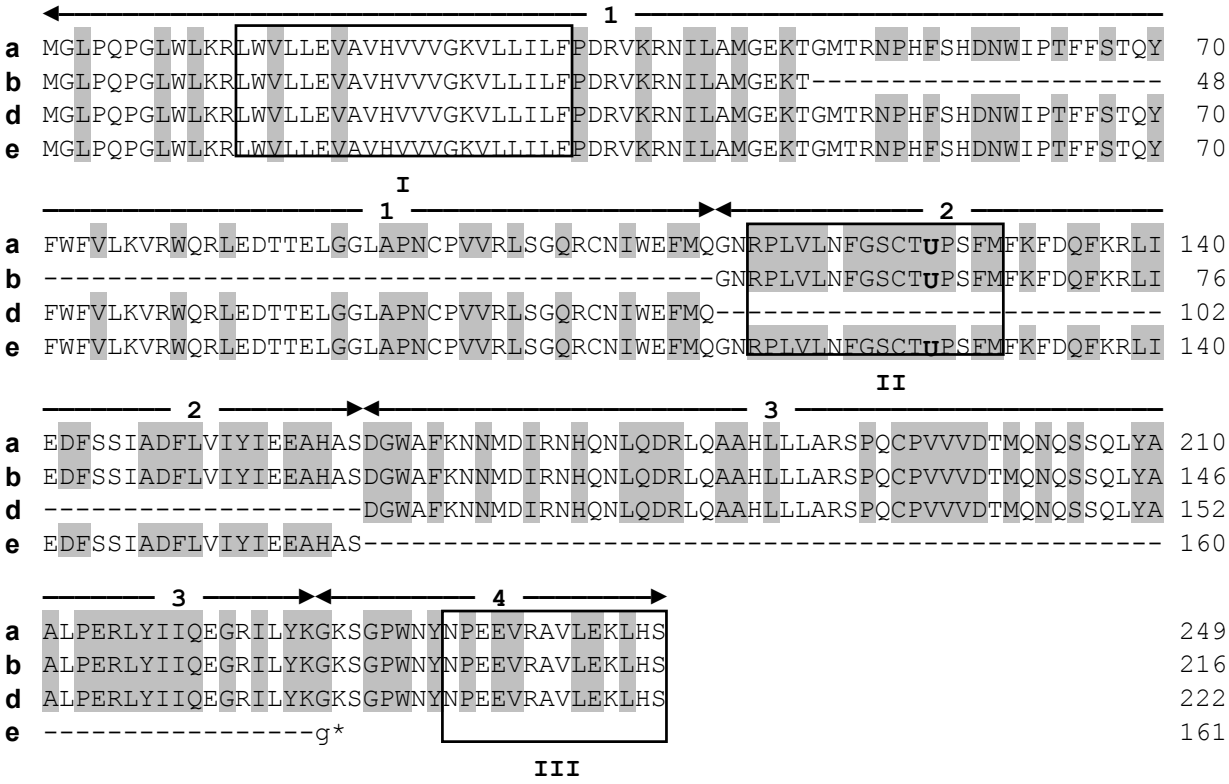

Fig. 5. Amino acid sequence of hD1 variants. The Sec is indicated by an U. Box I represents the transmembrane region, box II the catalytic center, whereas box III indicates the carboxyterminus of the variants, where the epitope of the antiserum used is located.

Table 1. Summary of the different splice variants identified in this study

Variant	Exons deleted	Number of nucleotides[a]	Number of amino acids	Domains conserved[b]			Number of clones	Accession number
				I	II	III		
a (wt)	-	750	249	+	+	+	24	AY560374
b	1CDE	558	185	+	+	+	2	AY560375 CD014030
c	1E,3	441	109	+	-	-	1	AY560376
d	2	606	201	+	-	+	1	AY560377 CD014029
e	3	550	161	+	+	-	9	AY560378 BG426442 BC017955 CK473178[c]
f	1E	641	109	+	-	-	5	AY560379 CD014031
k	1BC	626	40	+	-	-	1	AY560380
l	2,3	406	113	+	-	-	4	AY560381
m	1CD	667	85	+	-	-	1	AY560382
s	1CD,2	523	85	+	-	-	1	AY560383

a) including stop codon; total length of PCR products including primers is 28 nts larger
b) I, transmembrane domain (13-33); II, core sequence with Sec (115-129); III, C-terminal epitope (236-249)
c) rat D1 EST sequence

Table 1 provides a summary of the different splice variants identified in our study. Among other things, it indicates to what extent different domains have been conserved in the predicted proteins, such as the putative transmembrane domain, the catalytic core sequence, and the epitope recognized by our human D1 antibody (see also Fig. 5). Whereas the putative transmembrane domain is conserved in all splice variants, the Sec-containing catalytic center is only conserved in variants b and e, and the C-terminal epitope only in variants b and d. However, only variant a (wt hD1) protein was detected by immunoblotting of human liver, thyroid, and kidney microsomal fractions using the C-terminal antibody, suggesting negligible production of variant b and d proteins in these tissues (data not shown).

In addition to the genuine splice variants described above, our cloning experiments also resulted in a number of other variants apparently produced by deletion of the sequence between two identical short stretches of 2-6 nts which did not conform to the consensus splicing rules (Fig. 6). Figure 7 shows as an example the sequence of one of these variants (n), which was observed twice in our cDNA collection. It is unknown if these variants also occur naturally or if they are artificial products of the PCR and cloning techniques used.

```
                                        q
1   ATGGGGCTGCCCCAGCCAGGGCTGTGGCTGAAGAGGCTCTGGGTGCTCTTGGAGGTGGCT
                                          o   t
61  GTGCATGTGGTCGTGGGTAAAGTGCTTCTGATATTGTTTCCAGACAGAGTCAAGCGGAAC
121 ATCCTGGCCATGGGCGAGAAGACGGGTATGACCAGGAACCCCCATTTCAGCCACGACAAC
                                                          r
181 TGGATACCAACCTTTTTCAGCACCCAGTATTTCTGGTTCGTCTTGAAGGTCCGTTGGCAG
                p       j                       n
241 CGACTAGAGGACACGACTGAGCTAGGGGGTCTGGCCCCAAACTGCCCGGTGGTCCGCCTC
301 TCAGGACAGAGGTGCAACATTTGGGAGTTTATGCAAGGTAATAGGCCACTGGTGCTGAAT
361 TTTGGAAGTTGTACCTGACCTTCATTTATGTTCAAATTTGACCAGTTCAAGAGGCTTATT
                                    t
421 GAAGACTTTAGTTCCATAGCAGATTTTCTTGTCATTTACATTGAAGAAGCACATGCATCA
481 GATGGCTGGGCTTTTAAGAACAACATGGACATCAGAAATCACCAGAACCTTCAGGATCGC
                r       j       o                       p
541 CTGCAGGCAGCCCATCTACTGCTGGCCAGGAGCCCCCAGTGCCCTGTGGTGGTGGACACC
                                    n
601 ATGCAGAACCAGAGCAGCCAGCTCTACGCAGCACTGCCTGAGAGGCTCTACATAATCCAG
                                                        q
661 GAGGGCAGGATCCTCTACAAGGGTAAATCTGGCCCTTGGAACTACAACCCAGAGGAAGTT
721 CGTGCTGTTCTGGAAAAGCTCCACAGTTAA
```

Fig. 6. Generation of the hD1 variants j, n, o, p, q, r and t by deletion of sequence between two identical stretches of 2-6 nts which does not conform to the consensus splicing rules.

```
1     ATGGGGCTGCCCCAGCCAGGGCTGTGGCTGAAGAGGCTCTGGGTGCTCTTGGAGGTGGCT    60
61    GTGCATGTGGTCGTGGGTAAAGTGCTTCTGATATTGTTTCCAGACAGAGTCAAGCGGAAC   120
121   ATCCTGGCCATGGGCGAGAAGACGGGTATGACCAGGAACCCCCATTTCAGCCACGACAAC   180
181   TGGATACCAACCTTTTTCAGCACCCAGTATTTCTGGTTCGTCTTGAAGGTCCGTTGGCAG   240
241   CGACTAGAGGACACGACTGAGCTAGGGGGTCTGGCCCCAAACTGCC                 286
                                                  |
633                                               ACTGCCTGAGAGGCTCTACA   652
653   TAATCCAGGAGGGCAGGATCCTCTACAAGGGTAAATCTGGCCCTTGGAACTACAACCCAG   712
713   AGGAAGTTCGTGCTGTTCTGGAAAAGCTCCACAGTTAA                         750
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Fig. 7. Example of a variant (n) apparently produced by deletion of sequence between 2 identical short stretches (ACTGCC)

DISCUSSION

Recent genome-wide analysis has indicated that the transcripts of about half of the human genes are subject to alternative splicing. This indicates that splice variation is one of the most significant factors in the functional complexity of human genomic output (33, 41). With regard to iodothyronine deiodinases, alternative splicing has recently been demonstrated for human, mouse, chicken and juvenile lungfish (Neoceratodus forsteri) D2 (35, 36, 42, 43), but our study is the first to report on splice variation of the human D1 transcript. In both qualitative and quantitative terms, similar D1 splice variation was observed in different tissues (liver, kidney, thyroid) irrespective of age (fetal vs. adult). In addition to wt hD1 mRNA, evidence was obtained for the existence of at least 9 splice variants, 4 of which corresponded to sequences deposited in GenBank for cDNAs cloned from human liver and kidney. We also identified 2 aberrant D1 cDNA sequences in GenBank that had been cloned from human testis (variants h and i, accession codes AV661590 and AV661636, respectively). These variants were apparently produced by transcription initiation from additional upstream exons (A,B). In addition, variant i includes a sequence of 210 nts from an additional exon (C) located in intron 1 (Fig. 8). This additional sequence contains 2 in-frame TGA codons, potentially coding for Sec, and in-between a true TAG stop codon. Variants h and i lack the 5' end of exon 1 and, thus, could not be amplified by our PCR method using a forward primer located over the translation start codon. It remains unknown, therefore, if such hD1 mRNA variants will also exist in human liver, kidney and thyroid. However, we did not obtain any cDNA clone in this study that includes sequence from exon C.

Splicing is not a random process as intronic GT and AG pairs at the 5'and 3' splice junctions, respectively, are highly conserved; i.e. >99% of splice sites follow this consensus (44). All splicing events underlying the formation of mRNA for wt hD1 and the variants b, c, d, e, f, k, l, m and s also obey this GT-AG consensus. It is remarkable that the AGGT site at the exon 1DE junction is used as both donor splice site in variants c and f and acceptor splice site in variants m and s.

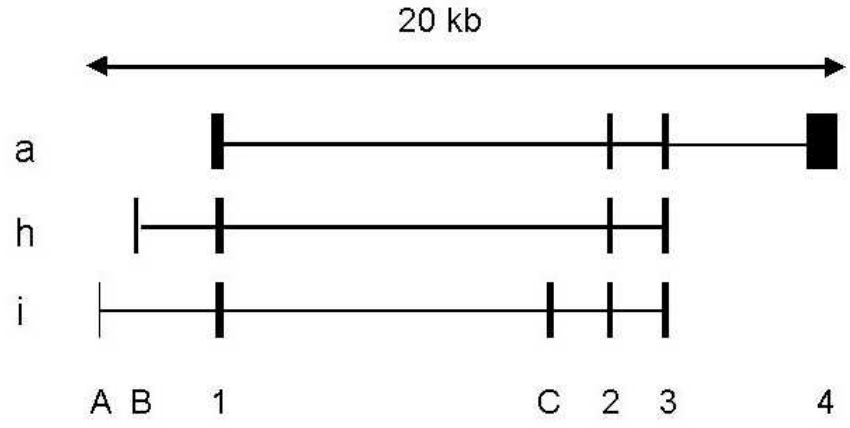

Fig. 8. Variant D1 sequences cloned from human testis (variants h and i). These variants were apparently produced by transcription initiation from additional exons upstream from exon 1 (A,B). Moreover, variant i also includes sequence from an additional exon (C) located in intron 1

The functional impact of splice variants is relatively unknown. Recent studies indicate that 70-88% of alternative splices change the protein product (30, 32, 33, 44). The majority of these changes seem to be functionally interesting, like replacement of the N or C terminus, or in-frame addition or removal of functional units (44). However, alternative splicing may be most important in complex systems where information must be processed differently at different stages (such as in the immune system) or where a very high level of diversity is required (such as in the CNS) (33). With regard to the relevance of the D1 splice variants identified in this study, a number of aspects should be considered: quantity, regulation and function of variant proteins.

Roughly half of the D1 cDNA clones obtained in this study represents wild-type mRNA and the other half represents splice variants, suggesting that alternative splicing has a large impact on the level of full-length mRNA and, thus, on production on functional D1 protein. However, it should be realized that the cloning procedure involved might be associated with a bias towards smaller cDNA fragments that have a higher cloning efficiency. Furthermore, the specific PCR method used with primers located over the translation start and stop codons is associated with a bias towards transcripts containing both primer sequences. The relatively high abundance of in particular variants e and f in our cloning experiments is supported by the results obtained with the GeneScan method, as well as by the presence of corresponding sequences in GenBank. These findings suggest that alternative splicing of human D1 is an

important phenomenon as it diminishes the amount of full-length mRNA. The lack of detection of variants b and d by immunoblotting of human liver extracts suggests that expression of the variant proteins is negligible. In combination with the severe mutilation of the D1 protein resulting from alternative splicing, it appears unlikely that the variant proteins have important biological functions as such. It is not excluded, however, that they may dimerize with wild-type subunits and thus affect the enzymatic properties of the latter.

The possible regulation of the alternative splicing of D1 transcripts in human tissues remains to be investigated. However, the very similar patterns of cDNA products obtained by RT-PCR of human liver, kidney and thyroid mRNA from adult and fetal subjects strongly suggests that alternative splicing is not affected by tissue-specific or age-dependent factors. However, it is not excluded that alternative splicing contributes to the down-regulation of hepatic D1 expression observed in patients with severe illness or in hypothyroidism.

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CHAPTER 5

INDUCTION OF THYROID-HORMONE DEGRADING DEIODINASE IN CARDIAC HYPERTROPHY AND FAILURE

Frank Wassen, Anja Schiel, George G.J.M. Kuiper, Ellen Kaptein, Onno Bakker, Theo J. Visser and Warner S. Simonides

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ABSTRACT

The similarities between the changes in cardiac gene expression in pathological ventricular hypertrophy and hypothyroidism suggest a role of impaired cardiac thyroid-hormone (TH) action in the development of contractile dysfunction during chronic cardiac pressure overload. Here we studied the possible involvement of altered cardiac TH metabolism using a rat model of right-ventricular (RV) hypertrophy induced by pressure-overload. Pathological RV hypertrophy was indicated by decreased mRNA levels of sarcoplasmic reticulum (SR) Ca^{2+}–ATPase type 2a (SERCA2a) and myosin heavy chain α (MHCα), and increased levels of MHCβ mRNA. Enzyme activity of type III deiodinase (D3), which converts T_4 and T_3 to the inactive compounds rT_3 and 3,3'-T_2, respectively, was identified in ventricular tissue. This activity was stimulated up to five fold in hypertrophic RV, but remained unaltered in the non-hypertrophic left ventricle (LV). A low level of type I deiodinase activity was also detected, which decreased significantly in both RV and LV. Stimulation of RV D3 activity was significantly higher in those animals in which hypertrophy progressed to heart failure, compared to animals that developed compensatory hypertrophy. The induction of a cardiac TH-degrading deiodinase may be expected to result in reduced cellular levels of T_3 and thereby contribute to a local hypothyroid state in the hypertrophic and, particularly, in the failing ventricle.

INTRODUCTION

Pathological ventricular hypertrophy caused by chronic overload of the heart is

characterized by changes in expression levels of contractile proteins and enzymes involved in intracellular Ca^{2+} regulation. The latter effects are a primary cause of the systolic and diastolic dysfunction seen in pathological hypertrophy. Progressive deterioration of Ca^{2+} homeostasis is thought to be critical in the transition from compensatory hypertrophy to heart failure (1). For many key enzymes the observed changes are similar to those induced by hypothyroidism, e.g., a shift to slower contractile proteins (MHCα to MHCβ, particularly in rodents), repression of SERCA2a and the SR Ca^{2+}-release channel (ryanodine receptor) and up-regulation of the sodium-calcium exchanger and phospholamban (2). An impairment of TH signalling has been suggested as a factor in re-directing gene expression. This is supported by the recent findings of diminished expression of nuclear TH receptors (TR) in hypertrophic rat hearts (3). Altered conversion or degradation of TH in the cardiomyocyte could be another mechanism resulting in reduced local biological activity of TH in pathological hypertrophy. Here we analyzed the activities of the three known iodothyronine deiodinases as a possible factor in altering the tissue thyroid state of the hypertrophic and failing heart. We show for the first time that D3 activity is present in ventricular tissue, and that this TH-degrading activity is upregulated in pathological hypertrophy.

MATERIALS AND METHODS

Animals

Animals were treated according to the national guidelines and with permission of the Animal Experimental Committee of the VU Medical Center Amsterdam, The Netherlands. Male Wistar rats, weighing 170-190 g (Harlan, Zeist, The Netherlands) were housed individually (250 cm^2/animal) and received food and water *ad libitum*. Animals were randomly assigned to the treated (n=30) or control (n=20) group, and given a single subcutaneous injection of either monocrotaline (MCT) (40 mg/kg) or saline. Animals were euthanized with an overdose of halothane after four weeks.

Deiodinase assays

Ventricular tissue homogenates were prepared and enzyme activities of deiodinase subtypes D1, D2 and D3 were performed as described previously (4).

mRNA analysis

Total RNA was isolated using the RNAzol B method (Campro Scientific, Veenendaal, The Netherlands). Serial dilutions of RNA were applied to nylon membranes (Hybond N^+, Amersham) using a vacuum slot-blot system (BioRad, Veenendaal, The Netherlands). Blots were hybridized with [α^{32}P]dCTP-labelled cDNA probes for SERCA2a, MHCα, MHCβ and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Membranes were exposed to phosphor imager screens, scanned and analyzed with ImageQuant software (Molecular Dynamics).

Analysis of plasma thyroid hormone levels

Plasma T_4, freeT_4 (fT_4), T_3 and TSH levels were determined using specific RIA's as described before (5).

Statistical analysis

Data are presented as means ± SEM. One-way analysis of variance was performed followed by Bonferroni comparison for post hoc analysis using Prism 3.0 software (GraphPad). Differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

MCT-induced right ventricular hypertrophy and cardiac gene expression

A single dose of MCT induces pulmonary vasculitis resulting in chronic pulmonary hypertension, affecting only the right side of the heart (6). The MCT-treated rat is consequently used as a model for pressure-overload induced RV hypertrophy and ventricular failure (7). In agreement with earlier studies (8,9) we found that approximately half of the animals developed RV hypertrophy (HYP) without signs of heart failure (n=16), whereas in the remaining animals (n=14) the RV hypertrophy progressed to severe congestive heart failure (CHF). The animals in the latter group started loosing weight around day 15 and showed pleural effusion and ascites at the time of sacrifice. The degree of RV hypertrophy was significantly higher in the CHF-group compared to the HYP-group, as indicated by the ratio of RV over LV+septum weight. Compared to controls this parameter was 2.5-fold higher in the CHF-group and 2-fold higher in the HYP-group (Table 1).

Table 1: Body weight (BW), ratio of RV over LV plus septum wet weight and plasma thyroid hormone values of all groups.

	Controls (n=20)	HYP (n=16)	CHF (n=14)
BW (g)	359 ± 5.5	325 ± 5.4 *	234 ± 6.5 *, ††
Ratio	0.21 ± 0.00	0.41 ± 0.02 *	0.50 ± 0.01*, ††
T_4	51.3 ± 2.8	39.8 ± 4.1	27.0 ± 3.4 *, †
fT_4	28.5 ± 0.9	21.1 ± 3.5	10.5 ± 2.2 *, †
T_3	2.12 ± 0.09	2.02 ± 0.07	1.19 ± 0.14 *, ††
TSH	1.73 ± 0.20	1.57 ± 0.15	2.18 ± 0.26

Values are means ± SEM, n = number of animals per group. T_4 and T_3 in nmol/L, fT_4 in pmol/L and TSH in ng/ml. * = $p < 0.001$ versus controls, † = $p < 0.05$ and †† = $p < 0.001$ versus HYP.

The wet/dry ratio of either ventricle was the same in all groups. There was no indication of LV hypertrophy in MCT-treated rats (data not shown). As indicators of the re-direction of gene expression in pathological hypertrophy we determined the RV mRNA levels for the MHC isoforms and SERCA2a. Figure 1 shows that the characteristic shift from the fast MHCα isoform to the slower MHCβ isoform was intermediate in the HYP-group, but almost complete in the CHF-group. Expression of SERCA2a mRNA was decreased by approximately 50% in both HYP- and CHF-rats.

Similar results concerning the degree of RV hypertrophy and SERCA2a mRNA levels have been reported for rats in which either compensatory hypertrophy or heart failure was induced by ligation of the pulmonary artery (10). There was no decrease in SERCA2a mRNA in LV of these animals and even though there was a shift in MHC isoforms in the CHF-group, it was much less pronounced than in RV (Fig.2).

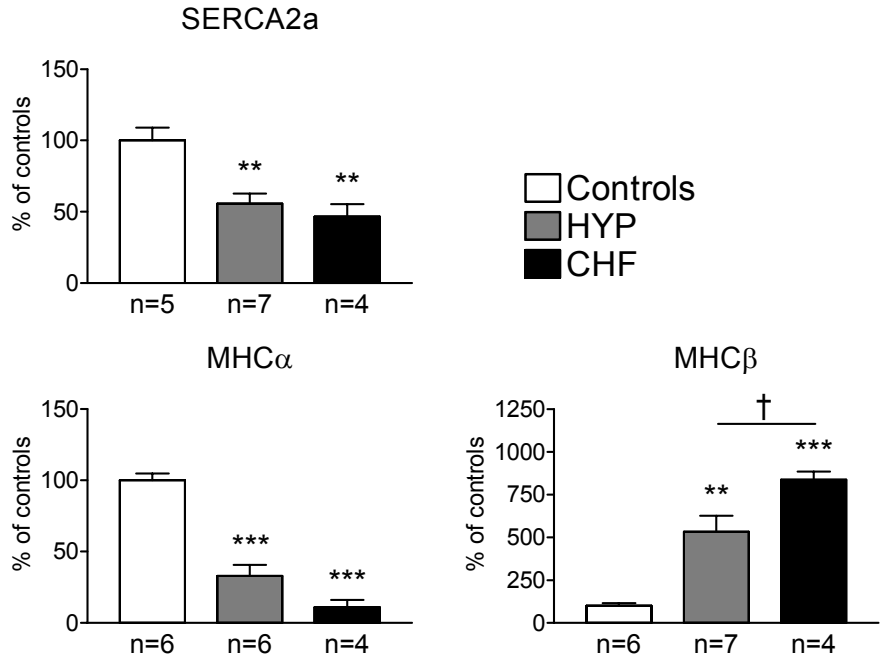

Fig. 1: SERCA2a and MHC isoform mRNA levels in right ventricles of treated and untreated rats. Data are means ± SEM and expressed as % of controls relative to GAPDH levels. ** = $p < 0.01$, *** = $p < 0.001$ versus controls, † = $p < 0.01$ HYP versus CHF.

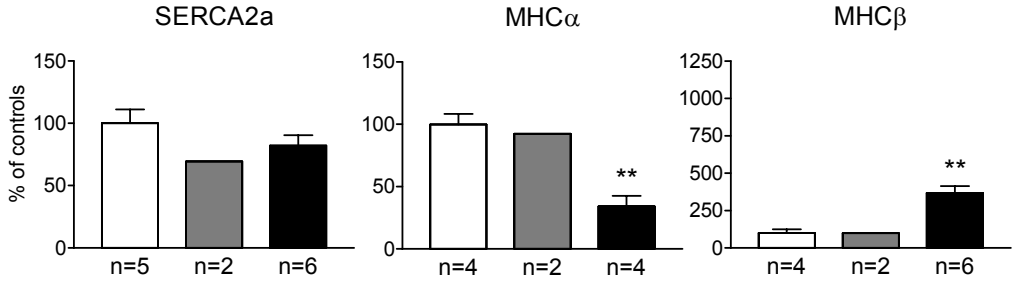

Fig. 2: SERCA2a and MHC isoform mRNA levels in left ventricles of treated and untreated rats. Data are means ± SEM and expressed as % of controls relative to GAPDH levels. ** = $p < 0.01$ versus controls. See Fig.1 for column legend

Plasma thyroid hormone levels

Plasma TH levels were measured at the time of sacrifice. Plasma T_4, fT_4, T_3 and TSH in the HYP-group were not different from control values indicating that the observed changes in T_3-responsive genes in compensatory hypertrophic RV are not due to systemic hypothyroidism. The CHF-group showed a significant 40-60% reduction of plasma T_4, fT_4 and T_3 with normal TSH levels (Table 1). Such changes are typical of serious, non-thyroidal

illness, including chronic heart failure (2). Although Ojamaa et al. recently showed that normalizing plasma T_3 levels in a rat model of LV hypertrophy following myocardial infarction restored the mRNA level of MHCβ, those of MHCα and SERCA2a were unaffected (11). Furthermore, using a rat model of pressure-overload induced LV hypertrophy, Kinugawa et al. reported a nearly complete shift in MHC isoform expression and a 50% reduction of SERCA2a mRNA in the absence of changes in plasma TH levels (3). These and our results, therefore, suggest that development of the phenotype of pathological hypertrophy, at least with respect to the MHC and SERCA2a genes, is largely independent of changes in plasma TH levels. However, an additional effect of reduced plasma TH levels in the CHF-group on these and other T_3-responsive genes may be expected in view of the LV data in Fig.2.

Expression of deiodinases

Apart from systemic levels, the intracellular availability of TH depends on cellular uptake rates of TH and on the intracellular metabolism of TH. Little is known about the cardiac expression of deiodinases and their possible role in determining TH levels in the heart. Low levels of D1 activity have been reported for cardiomyocytes from neonatal rats (12) and D2 mRNA has been found in the human heart (13), but enzyme activity has not been reported. Previous kinetic studies of cardiac iodothyronines in rats indicated no significant local T_3 production from T_4 (14), suggesting negligible activity of D1 or D2 and/or negligible cardiac uptake of T_4. The latter is supported by the recent observation that overexpression of D2 in cardiomyocytes of transgenic mice does not result in a considerable increase in cardiac T_3 levels (15). The significance of D1 or D2 activity and local cardiac T_3 production therefore seems limited and we directed our attention first to type III deiodinase, which converts T_3 and T_4 to the inactive compounds 3,3'-T_2 and rT_3, respectively (16). Many fetal tissues, as well as the placenta, express high levels of D3 activity, but in adult animals substantial levels of this enzyme have so far only been reported for the brain (16,17,18). Figure 3 shows the D3 activity that was readily detectable in ventricular homogenates, with similar levels in RV and LV from control rats.

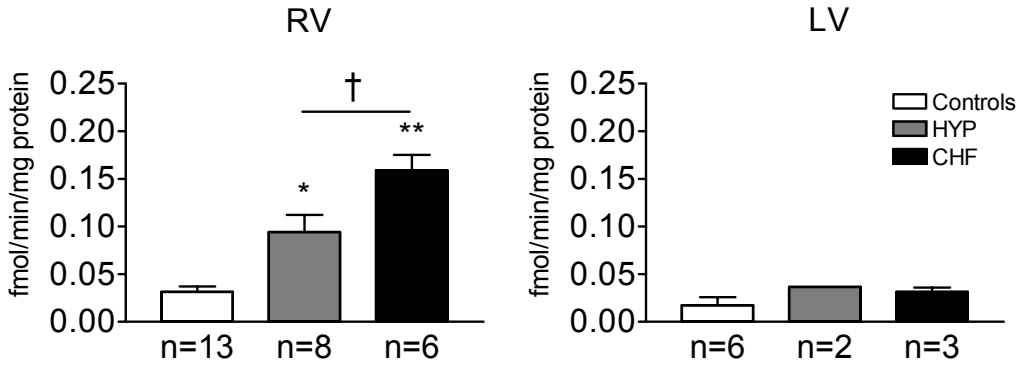

Fig. 3: Myocardial D3 activity in right and left ventricular homogenates. Data are mean ± SEM. ** = $p < 0.001$ and * = $p < 0.01$versus controls, † = $p < 0.01$ HYP versus CHF

The activity level increased 3-fold in hypertrophic RV and more than 5-fold in failing RV with no change in D3 activity in the non-hypertrophic LV of the same animals. Given this marked effect on D3 activity we determined the activity levels of the other deiodinases in these homogenates.

We failed to detect significant D2 activity in any of our samples but D1 activity was present, albeit at low levels (the specific activity in control ventricles was approximately 0.1% of that in the livers of these rats). In contrast to D3, the D1 activity was suppressed in the HYP- and the CHF-group (Fig.4). The similar reduction observed for RV and LV suggests a systemic effect on D1 activity in MCT-treated rats, rather than the hypertrophy-specific effect observed for the D3 activity. Gene expression of both D1 and D3 is positively regulated by T_3 (16) and it cannot be ruled out that the low plasma TH levels contribute to the down-regulation of D1 activity in the CHF-group. However, the reduction of D1 activity in the HYP-group, where plasma TH levels were normal, suggests a different mode of regulation. Given the already low D1 activity and the considerations presented in the previous paragraph, it is unlikely that the observed effect on this deiodinase will be of significance for cardiac TH metabolism.

The RV D3 activity in the CHF-group is about 10% of that in the brain of these animals. Whether this is sufficient to affect cardiac T_3 levels remains to be established, but preliminary analysis of RV tissue T_3 content indicate significant reductions of 30% in the HYP-group and more than 50% reduction in the CHF-group (M-J. Obregon and W.S. Simonides, unpublished observation). The HYP data are of particular relevance since plasma TH levels are still normal in these animals. D3 is expressed in the fetal stage of different tissues (18) and in non-thyroidal illness (17), and in a recent study we also

detected significant D3 activity in the fetal human heart, but very little activity in the adult human heart (R. Hume and T.J. Visser, unpublished observation). The induction or increased expression of various genes typical of the fetal stage is a characteristic aspect of pathological hypertrophy (19), and the enhanced RV-specific expression of D3 in our model of pressure-overload hypertrophy and failure may be part of such a growth program. Nonetheless, since our experiments were performed in whole tissue homogenates we cannot exclude the possibility that cells other than cardiomyocytes contribute to the measured activity. In two experiments we assessed ventricular D3 mRNA levels in the three groups by RT-PCR and the results indicated strong upregulation of this message in RV only, in parallel with the observed D3 enzyme activity (data not shown). This suggests that the observed regulation of deiodinase activity is at least in part pre-translational.

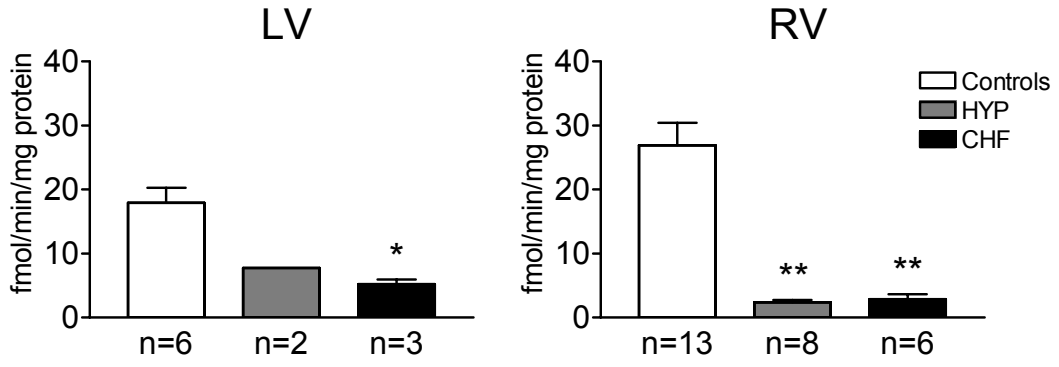

Fig. 4: Myocardial D1 activity in right and left ventricular homogenates. Data are mean ± SEM. * = $p < 0.05$, ** = $p < 0.001$ versus controls. Note that the absolute activities of D1 and D3 cannot be compared because of the different assay conditions for both enzymes.

Our findings do not negate the importance of possible defects in the T_3 signal transduction system, such as the recently reported down-regulation of TRs in chronic LV-pressure overload in rats (3). However, whether this is enough to create a local hypothyroid-like condition affecting T_3-responsive genes is not certain, since the full effect of hypothyroidism depends on the presence of TRs, which actively repress transcription of TH-responsive genes in the absence of hormone. As also noted in Ref. 3, the bioavailability of T_3 is, therefore, an essential factor determining the thyroid state of the cardiomyocyte. The present data suggest that increased D3 activity in pathological hypertrophy may lead to a reduction of local intracellular T_3 and as such contribute to a hypothyroid condition in the affected myocardium.

ACKNOWLEDGEMENTS

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CHAPTER 6

VENTRICULAR TYPE III IODOTHYRONINE DEIODINASE EXPRESSION AND T_3 CONTENT DURING DEVELOPMENT OF CARDIAC HYPERTROPHY AND FAILURE

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ABSTRACT

Changes in the ventricular expression of thyroid hormone (TH) responsive genes in cardiac hypertrophy and heart failure suggest an impairment of cardiac TH action. Using a rat model of right ventricular (RV) hypertrophy we recently described the RV specific induction of the TH-degrading deiodinase type III (D3) in end-stage heart failure. We now performed a time series analysis of cardiac gene expression, deiodinase activities, and tissue TH levels in RV and left ventricles (LV) during the development of either compensatory hypertrophy (HYP) or congestive heart failure (CHF) (0-4 wks). Deiodinase activities were unaltered in LV of any experimental group. In contrast, D3 activity increased more than ten fold in RV of CHF-hearts at three and four weeks. Ventricular T_3 and T_4 content decreased in RV and LV of CHF hearts at four weeks, reflecting reduced plasma TH levels. However, the T_3 content decreased to a significantly greater extent in RV of CHF-hearts only. Changes in mRNA levels of TH-responsive genes were most pronounced between weeks two and four in RV of CHF hearts. These data suggest that induction of D3 activity contributes to a more pronounced local hypothyroid state in the failing ventricle.

INTRODUCTION

Cardiac hypertrophy develops in response to a variety of pathological stimuli, and may eventually progress from a compensatory state into heart failure when the chronic overload is sufficiently high. Hypertrophy and congestive heart failure are accompanied by characteristic changes in the expression of numerous cardiac genes (1,2), resulting in an impairment of Ca^{2+} handling and contractility. For instance, changes in the expression of the sarcoplasmic reticulum Ca^{2+}-ATPase 2a (SERCA2a) and its regulatory molecule phospholamban (PLB) as well as of the myosin heavy chain (MHC) isoforms MHCα and MHCβ are thought to be critical for the systolic and diastolic dysfunction seen in heart failure. Expression of these genes is known to be regulated by thyroid hormone (TH) (3,4), as are a number of other cardiac genes implicated in heart failure, such as β_1-adrenergic receptors, G_i-protein subunits, voltage-gated potassium channels and the Na^+/Ca^{2+} exchanger (5,6). Because of the similarities in the pattern of gene expression in the diseased heart and the hypothyroid state, a role for TH in the development of cardiac hypertrophy or heart failure has been suggested (7-9).

The genomic actions of TH require the binding of T_3 to TH receptors (TR) associated with TH response elements in the promoters of TH responsive genes. Cellular uptake of T_3 (10) as well as the abundance of TRs are therefore important parameters in cardiac gene regulation, and changes in cardiac TR expression have indeed been found in human and rat studies of heart failure (7,8). Another mechanism potentially capable of influencing the cardiac activity of TH is the local metabolic conversion of T_4 or T_3 by deiodinases. However, cardiac uptake of T_4 and conversion to T_3 by outer ring deiodination by type I (D1) or type II deiodinase (D2) appears to be negligible (11,12). In agreement with this we detected very low ventricular D1 activity and no D2 activity in a recent study using a rat model of pressure overload induced right ventricular (RV) hypertrophy and end stage heart failure (13). Unexpectedly, however, we found a strong induction of the TH degrading deiodinase type III (D3) in the RV of failing hearts. This enzyme catalyses the inner ring deiodination of T_3 and T_4 to the inactive compounds 3,3'-diiodothyronine (3,3'-T_2) and rT_3, respectively. D3 activity has been detected in several tissues during embryonic development but in the adult situation substantial amounts of D3 are detected only in the brain, pregnant uterus and skin (14,15). D3 is considered to be an oncofetal gene because it was also found in monkey hepatocarcinoma cells (16) and is

upregulated by growth factors (17). This aspect of D3 regulation is interesting since the re-expression of a number of fetal genes is a common feature of cardiac hypertrophy (7,18). We hypothesized that the induction of D3 results in a cardiac specific reduction of T_3 and that such a decrease could contribute to the changes in gene expression seen in RV of hypertrophic hearts, but particularly in RV of failing hearts (13). In that study we analyzed hearts four weeks after the induction of hypertrophy, when animals had either developed compensated hypertrophy or had progressed to severe heart failure. A conclusion could therefore not be drawn about a possible role of D3 induction in the earlier stages of development of the failure phenotype.

In the present study we investigated the time course and relationship between systemic TH status, cardiac specific TH metabolism and their effect on cardiac TH levels and cardiac gene expression during the development of compensated hypertrophy (HYP) or congestive heart failure (CHF). We determined the mRNA levels of SERCA2a, MHC isoforms, PLB and atrial natriuretic factor (ANF) in right and left ventricles of rat hearts in which RV pressure overload was induced by a single injection of monocrotaline (MCT). The pyrrolizidine alkaloid MCT produces chronic pulmonary hypertension followed by the development of RV hypertrophy. We modified the existing experimental set-up to selectively and reproducibly induce either compensated hypertrophy or congestive heart failure by injecting animals with 30 or 80 mg of MCT, respectively. This enabled us to compare both groups at earlier time points, before critical differences become apparent.

MATERIAL AND METHODS

Animals

All animals were treated according to the national guidelines and with permission of the Animal Experimental Committee of the VU University Medical Center, Amsterdam, The Netherlands. Male Wistar rats, weighing 170-190g (Harlan, Zeist, The Netherlands) were randomly assigned to the three experimental groups.

Experimental protocol

After one-week acclimatization, animals received a single subcutaneous injection of MCT, 30 mg/kg (compensated hypertrophy = HYP) or 80 mg/kg (congestive heart failure = CHF). The control group was injected with saline. Animals were housed individually (250 cm^2/animal), on a 12h light/dark cycle and received food and water ad libitum.

Animals were weighed four times in the first two weeks and daily from day 14 onwards. Three animals (one of each group) died of unknown cause, 1 animal injected with 30 mg/kg was excluded because it showed signs of congestive heart failure. One hundred and fourteen animals (32 controls, 39 (30 mg) and 43 (80 mg) MCT treated) were included in the study. Two, three or four weeks after injection animals were killed with an overdose of halothane and the heart was rapidly excised. Blood was collected via the vena hepatica with heparin as anticoagulant. If necessary, first a trabecula was isolated, and the right ventricle (RV), left ventricle (LV) and septum were separated thereafter. All specimens were weighed and tissue was immediately snap frozen in liquid nitrogen. Material was stored at -80°C until further use.

Plasma and tissue TH

Plasma T_4 and T_3 were determined using specific RIA's as described before (19). Tissue TH concentrations were determined as previously described (20), using methanol-chloroform extraction, back extraction into an aqueous phase and purification of heart extracts on Dowex 1X2 columns. TH's were determined in the purified extracts using sensitive RIA's and results were calculated using individual recovery data obtained by the addition of tracer amounts of [^{131}I]T_4 and [^{125}I]T_3 to the initial homogenates.

RNA-isolation and Slot-Blots

Total RNA was isolated according to the method of Chomczynski and Sacchi (21). Briefly, frozen tissue was homogenized in RNAzol (Campro Scientific), RNA was isolated and quantified and the integrity was verified by gel-electrophoresis. Serial dilutions of RNA were applied to nylon membranes (Hybond N^+, Amersham) using a Vacuum Slot-Blot system (BioRad). Duplicate blots, containing RNA of all experimental groups, were then hybridized with cDNA probes for SERCA2a (22), MHCα and -β (23), ANF, PLB (24) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Hybridization with cDNA probes was performed overnight at 42°C in buffer containing 50% formamide, 5 x SSC, 5 x Denhardt's solution, 0.5% SDS and 50 μg/ml denatured salmon sperm DNA. The cDNA probes were labeled using the High prime labeling kit (Roche). α^{32}P-labelled dCTP was included in the reaction to obtain a specific activity of 1-5 x 10^8/μg DNA. Blots were washed in 2 x SSC/0.1% SDS and 0.1 x SSC/0.1% SDS at 42°C. Membranes were exposed to Phosphor imager screens, scanned, and analyzed using the ImageQuant

software (Molecular Dynamics). Quantification was performed by integrated optical density increase over background density in the rectangular region of interest. Data were expressed as the densitometric intensity of signals in arbitrary units relative to the GAPDH signal.

Contraction experiments

Trabeculae were isolated from RV's as described in detail before (25). All experiments were performed at 1mM external Ca^{2+} ($[Ca^{2+}]_o$). Briefly, hearts were removed and perfused with Tyrode buffer (120 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1 mM $CaCl_2$, 2 mM NaH_2PO_4, 24 mM $NaHCO_3$ and 10 mM glucose, pH 7.4, 20 mM 2,3-butanedione monoxime (BDM)), to remove any traces of blood. Long, thin, non-branched trabeculae were isolated and mounted in a superfused chamber (Tyrode buffer without BDM, 95% O_2/ 5% CO_2, 24°C) between a force transducer and a length adjustment device. Trabeculae were stretched to a length corresponding to a passive force of ~ 8% of the maximum force (F_{max}) which is about 87% of maximum length and equilibrated for 45 min with a stimulation frequency of 0.5 Hz; then recordings were taken of single twitches at 0.2 Hz.

Deiodinase assays

D1 and D3 activities were determined in ventricular homogenates as described previously (26). D1 activity was measured by the release of radioiodide by outer-ring deiodination of outer-ring labeled rT_3. Homogenates were incubated for 60 min at 37°C with 0.1 µM [3',5'-^{125}I]rT_3 for 60 minutes at 37°C in the absence or presence of 0.1 mM PTU in 0.1 ml 0.1M phosphate (pH 7.2), 2 mM EDTA, and 10 mM DTT. Reactions were stopped by adding 0.1 ml 5% ice-cold bovine serum albumin. The protein-bound ^{125}I-labeled iodothyronines were precipitated by addition of 0.5 ml 10% trichloroacetic acid. After centrifugation, the supernatants were analysed for ^{125}I–production on Sephadex LH-20 minicolumns (bed volume 0.25 ml), equilibrated and eluted with 0.1 M HCl. D3 activity was measured by analysis of the formation of the radioactive deiodination products 3,3'-T_2 and eventually 3'-iodothyronine from outer-ring labelled T_3 using high performance liquid chromatography (HPLC). Homogenates were incubated for 60 min at 37°C with 1 nM [3'-^{125} I]T_3 in the presence or absence of 0.1 µM T_3 in 0.1 ml 0.1 M phosphate (pH 7.2), 2 mM EDTA, and 50 mM DTT. Reactions were stopped by addition

of 0.1 ml ice-cold methanol. After centrifugation, 0.1 ml of 0.02 M ammonium acetate (pH 4) was added to 0.1 ml supernatant, and 0.1 ml of this mixture was applied to a 4.6 x 250 mm Symmetry C18 column connected to an Alliance HPLC system (Waters, Etten-Leur, The Netherlands), and eluted with a gradient of acetonitril in 0.02 M ammonium acetate (pH 4) at a flow of 1.2 ml/min. The proportion of acetonitril was increased linearly from 30% to 44% in 10 min. The radioactivity in the eluate was determined using a Radiomatic A-500 flow scintillation detector (Packard, Meriden, CT).

Statistical analysis

Data are presented as mean ± standard error if not stated otherwise. One-way ANOVA was applied for comparison between groups, followed by Bonferroni's multiple comparison post-hoc test. Differences were considered significant at $p<0.05$. Prism 3.0 (GraphPad) was used for the statistical analysis.

RESULTS

Development of hypertrophy and heart failure

With the exception of one animal, which was excluded from the analysis, rats treated with 30 mg/kg MCT showed no secondary signs of right-sided CHF such as liver cirrhosis, pleural effusion, ascites and weight loss at any time point during the four week experimental period. The RV hypertrophy developed by this group (see below) was therefore considered compensatory (HYP). In contrast, all animals treated with 80 mg/kg eventually showed clear signs of CHF. After two weeks no signs of heart failure were detectable in any MCT-treated group, while after three weeks some animals of the CHF-group developed the first signs of heart failure. CHF-rats started loosing weight between days 14-18, while HYP rats did not cease to grow over the entire four-week experimental period (Fig. 1). As a result, CHF animals had significantly lower body weights from day 10 on, compared to controls and after two and four weeks compared to HYP animals. The lung weight was significantly increased in both MCT-treated groups compared to controls, and significantly higher in CHF-rats compared to HYP rats after three and four weeks. This increase in lung weight is the result of proliferative pulmonary vasculitis (27) rather than edema formation considering that dry/wet weight ratios of the lungs were not different between the experimental groups at any time point (data not shown). There was

also a significant reduction in liver weight in CHF-rats but not in HYP-rats compared to controls after three and four weeks.

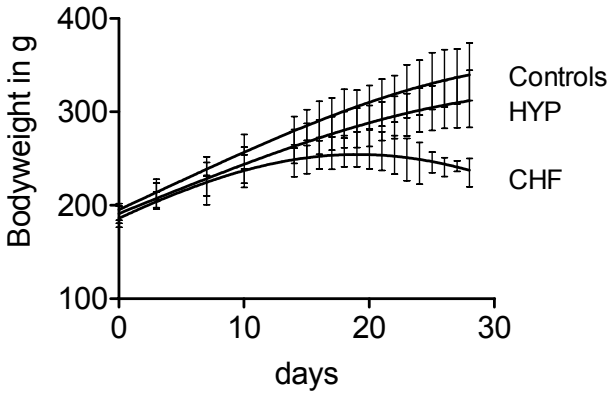

Fig. 1: Mean growth curves of control (n=12), HYP- (n=16) and CHF-rats (n=20), followed four weeks after MCT treatment. Values are mean ± SD. Differences were significant between CHF and controls from day 10 on ($p < 0.001$) and between HYP and CHF from day 20 ($p < 0.001$).

The liver weight was not different after two weeks, suggesting that the decreased liver weight was secondary to the development of heart failure, probably due to necrosis. RV weight and ratio of RV over left ventricle plus septum (LV+S) were significantly higher in both MCT-treated groups compared to controls already after two weeks, indicating the development of RV hypertrophy. After four weeks this ratio also differed significantly between the HYP- and CHF-groups. Organ weights are summarized in Fig. 2.

Hypertrophy and heart failure are associated with impaired functional properties of the myocard. Typically the rates of cardiac contraction and relaxation are lower due to changes in contractile protein composition and, particularly, due to reduced Ca^{2+} handling capabilities (28). To confirm the presence of such a functional impairment in our model we analyzed twitch characteristics in isolated RV trabeculae, four weeks after MCT treatment. Force recordings revealed a prolongation of twitches in both MCT treated groups (Fig. 3). The time to peak tension increased from 162 ± 7 ms in controls to 239 ± 8 ms in HYP and 221 ± 6 ms in CHF, while the time to half relaxation was significantly increased from 123 ± 9 in controls to 199 ± 9 in HYP and 157 ± 8 in CHF trabeculae (all $p < 0.05$ vs. controls).

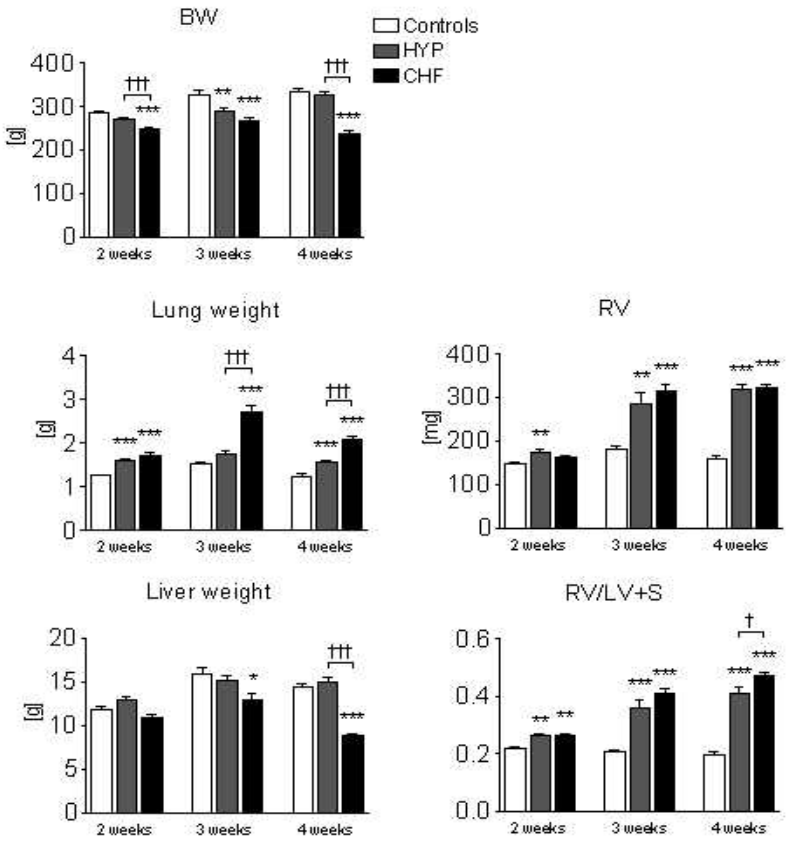

Fig. 2: Body weights (BW), right ventricular weights (RV), ratio of RV over left ventricle plus septum (RV/LV+S), lung weights and liver weights at two, three and four weeks after MCT injection. Data are mean ± SEM. * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$ versus controls same week, † = $p < 0.05$ and ††† = $p < 0.001$ versus HYP.

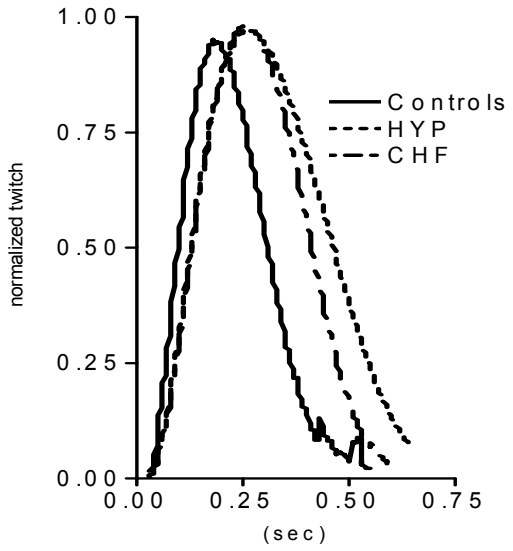

Fig. 3: Normalized twitch readings from control (n=11), HYP (n=12) and CHF (n=9) trabeculae.

Thyroid hormone status

We determined the plasma levels of T_3 and T_4 at two, three and four weeks after MCT treatment. We detected no significant decrease in T_3 or T_4 at any time point in the HYP group compared to controls. In contrast, both TH's decreased significantly in the CHF group compared to controls. This decline was gradual from three to four weeks resulting in a 60% drop in plasma T_3 and T_4 four weeks after MCT treatment. Results are summarized in Fig. 4.

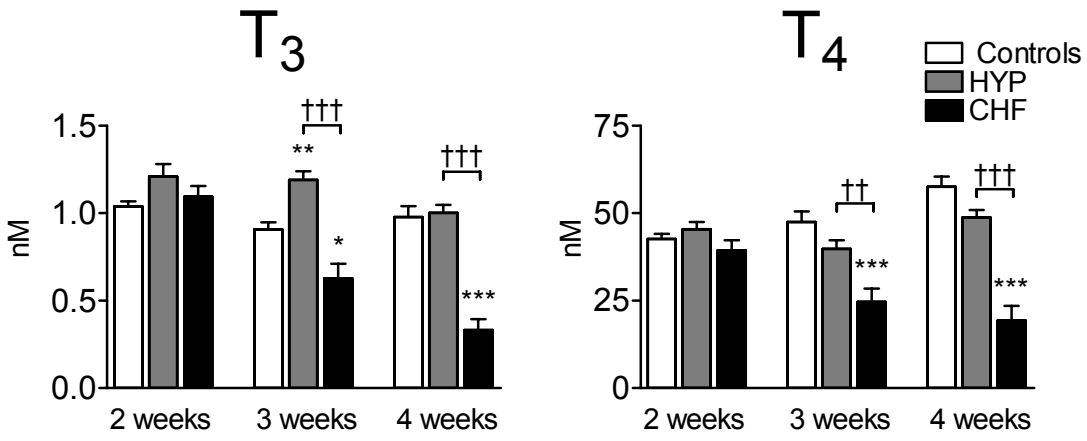

Fig. 4: Plasma T_3 and T_4 levels at two, three and four weeks after MCT treatment. Controls are n = 6 at two and four and n =8 at three weeks, HYP and CHF are n = 8 at all time points. Data are mean ± SEM. * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$ versus controls, same week and †† = $p < 0.01$ and ††† = $p < 0.001$ versus HYP same week.

Quantification of mRNA levels

To investigate if the expression of SERCA2a, PLB, the MHC isoforms and ANF were differently altered depending on the degree of hypertrophy we determined the mRNA levels of those genes in RV and LV tissue two and four weeks after MCT administration. Results for RV are summarized in Fig. 5 and those for LV in Fig. 6. ANF mRNA expression was analyzed as a general marker of hypertrophy. In RV of the CHF-group, its expression at two and four weeks after MCT administration increased 4.5 and 9 times, respectively. A similar trend was seen for the HYP-group, but this did not reach statistical significance. ANF mRNA remained unaltered at any time point in LV of both MCT-treated groups.

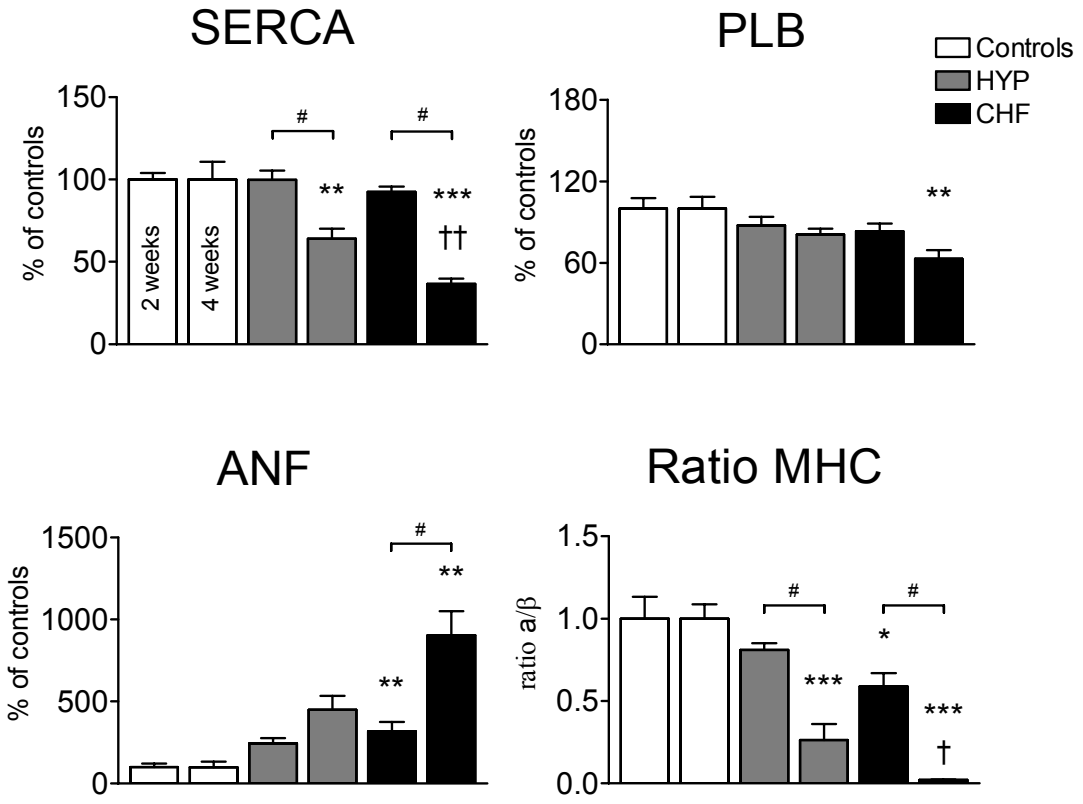

Fig. 5: RNA expression levels of SERCA (A), PLB (B), ANF (C) and the ratio of MHCα to MHCβ (D) in right ventricular total RNA two (left bars) and four weeks (right bars) after MCT treatment. Data are mean ± SEM. Number of experiments are: Controls n = 6 at two and n = 5 at four weeks, HYP n = 8 at two and n = 5-6 at four weeks and CHF n = 6-7 at two and n = 8-10 at four weeks. * = $p < 0.05$ ** = $p< 0.01$ and *** = $p< 0.001$ versus controls, and † = $p< 0.05$ and †† = $p< 0.01$ versus HYP. # = $p < 0.001$ vs 2 weeks

SERCA2a mRNA was unaltered in LV of the HYP-group at two and four weeks. In the CHF-group SERCA2a mRNA was unchanged after two weeks but was decreased by 30% after four weeks. In RV SERCA2a mRNA remained unaltered after two weeks in both MCT-treated groups, but decreased significantly after four weeks by 36% in the HYP-group and by 63% in the CHF-group.

PLB mRNA was unaltered in LV at any time point in both MCT-treated groups. In RV, PLB mRNA levels remained unaltered in the HYP-group at two and four weeks. Again, in RV of the CHF-group, PLB mRNA was unaltered at two weeks, but decreased by 36% after four weeks compared to controls ($p < 0.05$).

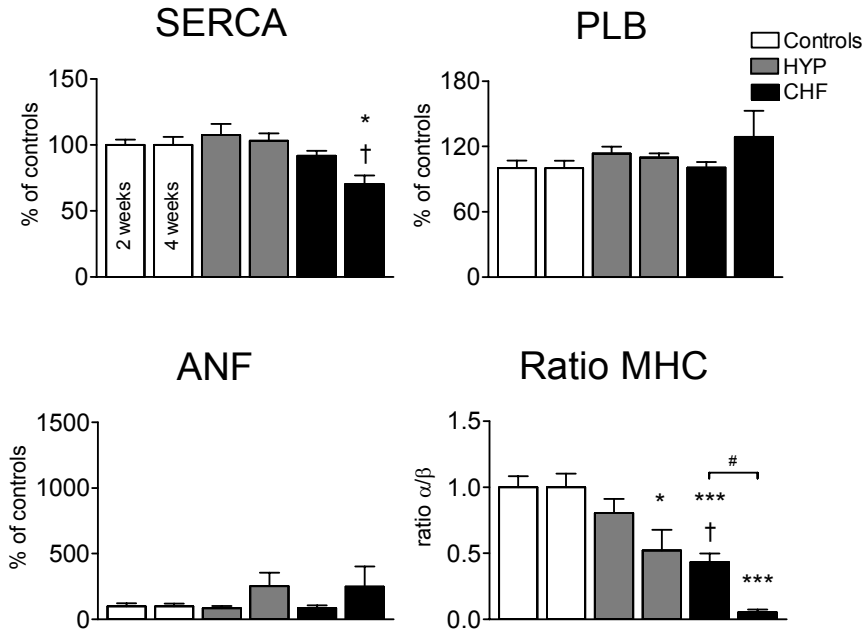

Fig. 6: RNA expression levels of SERCA (A), PLB (B), ANF (C) and the ratio of MHCα to MHCβ (D) in left ventricular total RNA two (left bars) and four weeks (right bars) after MCT treatment. Data are mean ± SEM. Number of experiments are: Controls n = 6 at all time points, HYP and CHF n = 7 at two and n = 4 at four weeks. * = $p < 0.05$ and *** = $p < 0.001$ versus controls, and † = $p < 0.05$ HYP. # = $p < 0.05$ versus 2 weeks.

There was a shift in the expression of the MHC isoforms from the MHCα to the MHCβ isoform. The ratio of MHCα to MHCβ mRNA was unaltered in LV and RV of the HYP-group after two weeks, but the ratio decreased significantly to 0.5 in LV and to 0.3 in RV at four weeks (control 1.0). In contrast the ratio was significantly decreased in LV and RV at all time points in CHF-hearts. After two weeks this ratio amounted to 0.4 in LV and 0.3 in RV, but after four weeks MHCα mRNA was almost completely absent in CHF-rats, with MHCα/MHCβ ratios as low as 0.06 in LV and 0.02 in RV.

Deiodinase activities

Deiodinase activities are depicted in Fig. 7. The D1 activity was detectable in cardiac tissue but was very low compared to what is found in liver (13). While there was no significant change in LV in the MCT-treated groups at any time point, the levels of D1 activity decreased significantly in RV of the HYP-group after four weeks ($p < 0.001$ vs. controls) and in RV of the CHF-group after three ($p < 0.01$) and four weeks ($p < 0.001$). A low D3 activity was detectable in RV and LV of controls and this did not change in LV of

either MCT-treated group. In contrast, RV D3 activities in the CHF-group increased more than ten fold after three ($p < 0.01$) and four ($p < 0.001$) weeks.

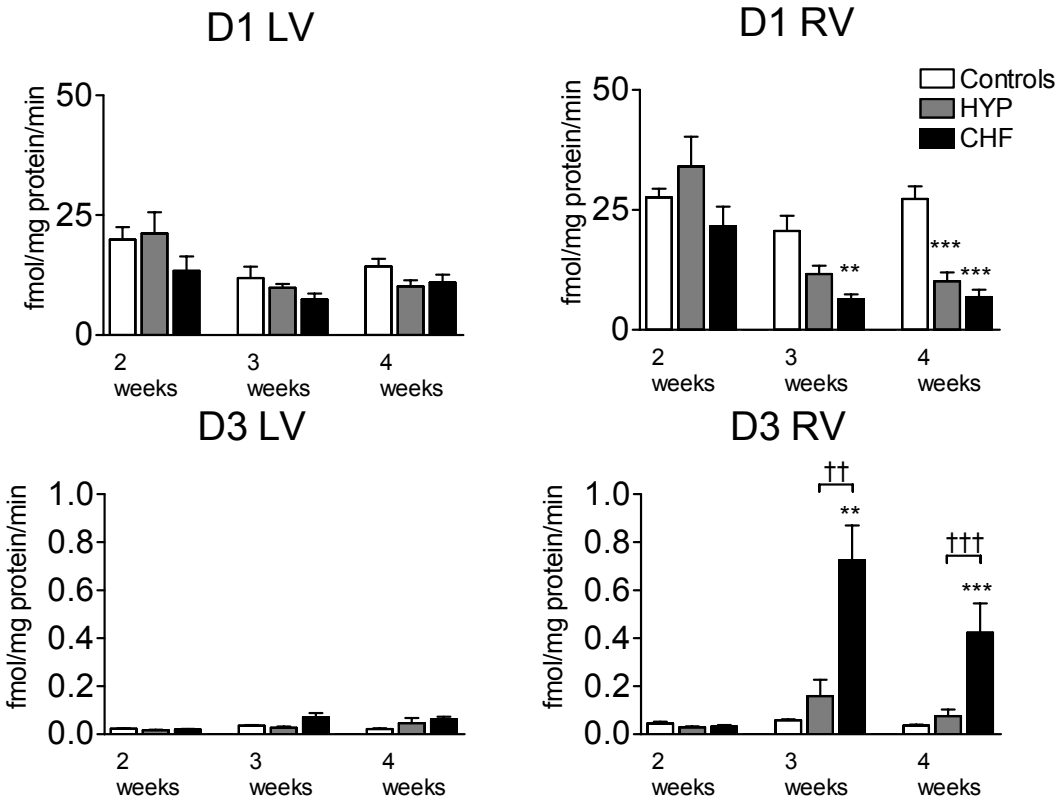

Fig. 7: Deiodinase Type I (D1) and III (D3) activities in whole tissue homogenates of left and right ventricles at two, three and four weeks after MCT injection. Controls are n = 5 at two, n = 4 at three and n = 13 for left and n = 20 for right ventricles at four weeks. HYP are n = 6 at two, n = 4 at three and n = 9 at four weeks. CHF are n = 6 at two, n = 4 at three and n = 7 at four weeks. Data are mean ± SEM. ** = $p < 0.01$, *** = $p < 0.001$ versus controls, †† = $p < 0.01$ and ††† = $p < 0.001$ versus HYP.

Tissue thyroid hormone levels

Tissue T_3 and T_4 content in LV and RV were determined four weeks after MCT treatment. In the HYP-group tissue T_3 and T_4 levels were not altered with the exception of a small decrease in RV tissue T_3. However, in the CHF-group tissue T_3 and T_4 levels were significantly decreased in both ventricles (Fig. 8). Furthermore, comparison of the reduction in T_3 content relative to that of T_4 in LV and RV indicates a significantly greater reduction in tissue T_3 in RV. This is indicated by the tissue T_3 to T_4 ratios presented in Fig. 9.

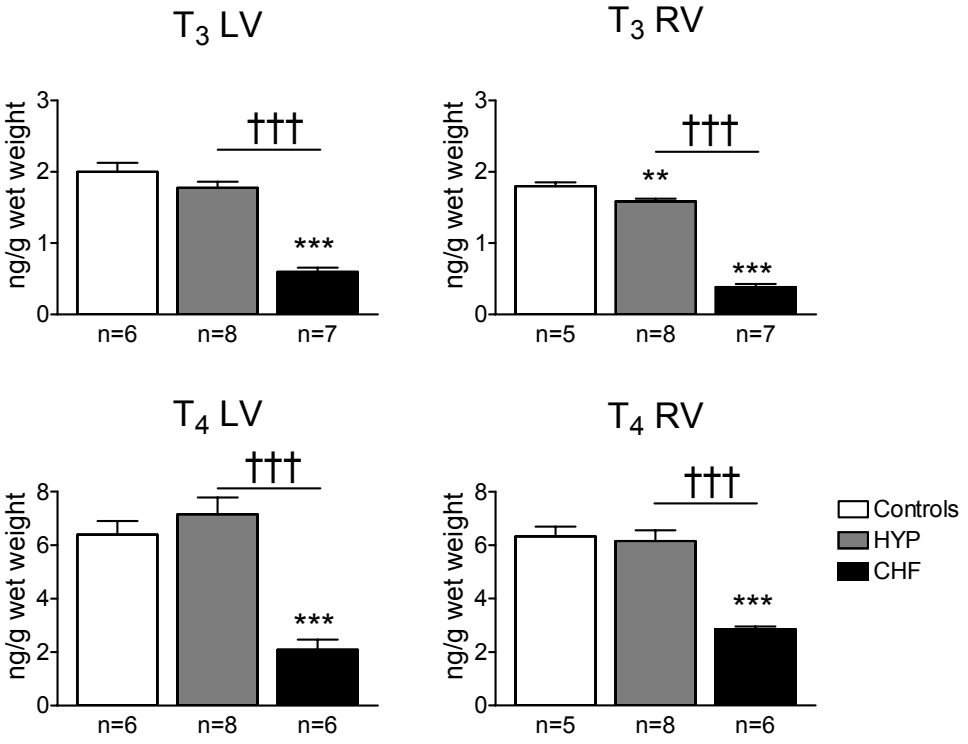

Fig. 8: Tissue T3 levels in LV (A) and RV (B) and tissue T4 levels in LV (C) and RV (D), four weeks after MCT administration. Data are mean ± SEM. ** = $p < 0.01$, *** = $p < 0.001$ versus controls and ††† = $p < 0.001$ versus HYP.

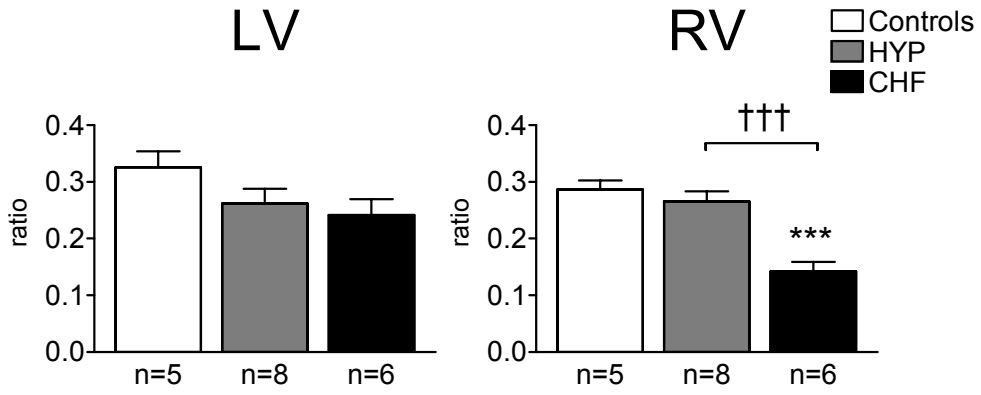

Fig. 9: Ratio of tissue T_3 over T_4 determined in whole tissue homogenates of LV and RV four weeks after MCT treatment. Data are mean ± SEM. *** = $p < 0.001$ versus controls and ††† = $p < 0.001$ versus HYP.

DISCUSSION

In this study we show an RV-specific increase in D3 activity in pressure overload induced right-sided heart failure, which is absent in animals that develop compensated hypertrophy. The dramatic induction of D3 does not appear to be part of the initial hypertrophic response, yet maximal D3 activity is already attained three weeks following induction of hypertrophy, when animals start showing secondary signs of heart failure. The determination of cardiac TH levels revealed an RV-specific decrease in the tissue T_3 content in CHF-hearts only. Analysis of the mRNA levels of important cardiac genes in LV and RV suggests the presence of two separate mechanisms affecting ventricular gene expression, *i.e.* a TH-independent mechanism, specific for pathological hypertrophy, and an additional TH-dependent mechanism, which becomes evident in CHF.

In our previous study we used 40 mg/kg MCT to induce pulmonary hypertension and RV hypertrophy, resulting in 50% HYP and 50% CHF animals (13). However, this distinction does not become evident until after three weeks and analysis of early events underlying the development of either HYP or CHF is therefore not possible. In the present study we used 30 mg/kg MCT, resulting in 100% HYP animals, or 80 mg/kg MCT, resulting in 100% CHF animals. This allowed us to study changes in tissue deiodinase and TH-sensitive gene expression during the development of compensated hypertrophy or heart failure. Comparison of data at four weeks from both studies shows that the two methods yield virtually identical HYP- and CHF-groups with respect to body weight, degree of RV hypertrophy and RV mRNA levels of SERCA2a and MHC-isoforms. The thyroid status of the respective groups was also comparable, with no change in plasma T_3 or T_4 levels in the HYP-group at any time, whereas the CHF-group developed signs of non-thyroidal illness (NTI). Animals in this group had decreased T_3 and T_4 levels from week three on and the drop in plasma levels was most pronounced at week four. This is in agreement with our earlier findings, which also showed that the ultimate 60% drop in plasma T_3 and T_4 was accompanied by a decrease in free T_4 and unaltered TSH levels (13).

The changes in mRNA levels of SERCA2a, its regulatory protein PLB and the shift from the fast MHCα to the slow MHCβ isoforms in RV of HYP- and CHF-hearts, suggest reduced Ca^{2+}-handling capabilities and altered contractile properties. This was confirmed by the twitch analysis of RV trabeculae, which showed lower rates of contraction as well

as relaxation, hallmarks of contractile impairment in cardiac hypertrophy. Yet, the prolongation of twitches did not differ between the HYP- and the CHF-group, even though changes in mRNA profile were more pronounced in the CHF-group at four weeks. It was recently shown that twitch characteristics of rat heart tissue are critically dependent on temperature (29) therefore the functional differences may not become apparent at the experimental temperature and stimulation frequency typically used in contraction analyses (24°C, 0.2 Hz).

The analysis of deiodinase activities confirmed the reciprocal regulation of D1 and D3 activity in RV's of CHF-hearts reported earlier (13) and showed a maximal effect already at three weeks, where no effect was seen at week two. In the HYP-group, only a small, not statistically significant increase in D3 activity was present. The RV-specific induction of D3 in of the CHF-group could be part of the re-expression of a fetal gene program that needs to be activated to allow cell growth (30). Yet, it does not appear to be part of the initial changes in gene expression, which become evident at two weeks. Furthermore, HYP hearts also develop hypertrophy and show some of the characteristic changes that are considered to be part of that re-expression pattern, without an induction of D3. It is likely that the pathological stimulus in the CHF-group is greater, or sustained over a longer period of time, and that not all genes that form the fetal pattern are activated at the same time. Alternatively, an additional stimulus might be responsible for the induction of D3 activity, which may be only activated when the overload is not successfully compensated and then initiates the transition into heart failure.

We do not know with certainty whether the deiodinase activity is located exclusively in cardiomyocytes or whether other cell types present in the heart contribute to the activity in whole tissue homogenates. Although the relevance of the reduction of the already very low D1 activity for local TH metabolism is most likely negligible, the substantial D3 activity in RV's of CHF-hearts was suspected to result in a change in local tissue TH levels. We therefore determined tissue T_3 and T_4 levels four weeks after MCT administration. Even though levels for both TH's decreased significantly in LV and RV, reflecting the reduced plasma TH levels, the tissue T_3 to T_4 ratio was significantly decreased in RV of the CHF-group only, compared to controls as well as to the HYP-group (Fig. 9). This is caused by a greater decrease in tissue T_3 in RV's of the CHF-group and we suggest that this is the effect of increased T_3 degradation by D3.

The effects on SERCA2a and MHC-isoform mRNA levels in the non-hypertrophic LV in the CHF-group show the effect of the reduced LV TH content on gene expression.

However, the MHC ratio is also significantly altered in LV of the HYP-group, where NTI is absent and ventricular TH levels are normal. Similar effects in LV in the absence of a direct pathological stimulus have been seen in this model in regard to alterations in the β-adrenoreceptor-adenylate cyclase system (31,32). These authors speculate that the changes in the LV are due to a neuro-endocrine overstimulation due to the right-sided heart failure.

The effects on gene expression in the RV depend on the severity of the underlying hypertrophy, as indicated by the increase in levels of ANF mRNA and the actual degree of RV hypertrophy. The decrease in SERCA2a expression is particularly relevant given its key role in myocardial function. The importance of SERCA2a has been proven by several studies demonstrating that prevention of the decrease in SERCA2a by adenoviral transfer improved myocardial function and prevented the development of heart failure (33-35). In HYP-hearts the decrease in SERCA2a mRNA expression is the result of the pathological stimulus and it is TH independent. Yet, the more severe decrease in RV SERCA2a mRNA in the CHF-group is most likely the result of a local decrease in tissue T_3 content, caused in part by the induction of D3 activity.

It is known that the abundance of the different TR isoforms is altered in the aging rat heart and the diseased human heart (7). It was therefore suggested that diminished TH signaling due to changes in TR expression could account for the gene expression pattern seen in heart failure, even under euthyroid conditions (9). In a rat model of cardiac hypertrophy it was shown that all TR isoforms were indeed decreased in their expression (8). This model did not show changes in plasma TH levels and is therefore comparable to our HYP-group. Because we have not determined the abundance of TRs in our model, we cannot exclude a contribution to the effects described here.

We conclude that altered TH metabolism, specifically increased T_3 degradation by D3, worsens the alterations in TH-dependent gene expression in the transition to heart failure by decreasing the levels of T_3 in the diseased ventricle. It is possible that the increase in D3 activity is part of the onset of the re-expression of a fetal gene program but its absence in HYP-hearts suggests a late activation only in response to a sustained pathological stimulus or the need for an additional, not yet identified, stimulus. We have shown here that altered TH metabolism is a possible contributor to the suppression of SERCA2a gene expression in severe heart failure and we propose that prevention of the decrease in ventricular tissue levels of T_3 could be beneficial in preventing myocardial impairment.

ACKNOWLEDGEMENTS

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CHAPTER 7

GENERAL DISCUSSION

I Structure-activity relationships of deiodinases

Feline D1 versus other D1 enzymes

The aim of the study described in chapter 2 was to investigate the molecular basis for the substrate selectivity of cat D1. As was reported earlier, cat D1 is an interesting enzyme, because of its weak activity towards nonsulfated substrates such as rT3 (1). This is in agreement with previous reports on native dog D1 as well as the wild-type recombinant enzyme expressed in HEK293 cells (2-5). Indirect evidence for this has been reported in another study (6), where the lower serum T4, T3 and rT3 binding in cats and dogs vs. humans is associated with significantly shorter total mean residence times as well as significantly more rapid serum clearance rates for the two former iodothyronines, whereas this was not observed for the latter. The explanation for this discrepancy given by the authors was that other factors were involved influencing both parameters. This could be in line with our observation on cat D1 and that of others on dog D1 (2) that rT3 is a poor substrate, because decreased degradation of rT3 observed in these species counteracts the effect of lower serum binding on residence and clearance times of rT3. Thus, the minor effects on rT3 kinetics despite the lower rT3 serum binding in dogs vs. humans could well be explained by poor deiodination of rT3 by D1 in dogs and probably also in cats, although data on rT3 binding in the cat are lacking. This is in contrast to rat and human D1 that has rT3 as the preferred substrate. After we confirmed the slow deiodination of rT3 by cat liver microsomes it was shown on Western blot that the expression level of cat D1 does not differ significantly from that in other species, like in pigs. Hereby we confirmed, that the slow deiodination of rT3 by cat D1, reflected by a high Km value, is an intrinsic property of cat D1 enzyme. Substrate preference of cat D1 (rT3S>T2S>T3S>>rT3) was similar to dog D1 (T3S>T2S>rT3) but differed significantly from human and rat D1 (rT3≈rT3S>T2S>T3S) as well as porcine D1 (rT3≈rT3S>T3S>T2S).

Table 1. Kinetic parameters of native mammalian D1 in liver microsomes.

Species	Residues[a]			Reaction	Km µM	Vmax pmol/min.mg	Vmax/ Km[c]	Ref.
	45-46	48-52	65-66					
Human	GE	TGMTR	FF	rT3 ORD	0.5	120	240	(7)
Porcine	SQ	TGMAK	FF	rT3 ORD	0.2	100	625	
Rat	GQ	TGMTR	FF	rT3 ORD	0.2	1520	6909	
Cat	NR	-	LY	rT3 ORD	11	54	4.9	
Human	GE	TGMTR	FF	rT3 ORD	0.2	242	1152	(8)
Human				T4 ORD	1.9	6.9	3.7	
Human				T4 IRD	3.4	6.5	1.9	
Human				T3 IRD	2.8	11.2	4.0	
Human				T3S IRD	2.4	244	102	
Human				T2S ORD	0.49	64	131	
Dog	NG	-	LY	rT3 ORD	5.9, 4.2	481, 278	74.3	(3)
Dog				T4 ORD	7.6, 7.5	188, 204	26	
Dog				T4 IRD	6.9, 12.9	52, 91	7.3	
Dog				T3 IRD	27.7, 27.7	228, 254	8.7	
Dog				T3S IRD	9.3, 7.4	7213, 7787	911	
Dog				T2S ORD	12.1 ± 4.2	6232 ± 2588	507	
Rat	GQ	TGMTR	FF	T4 ORD	2.3	30	13	(9)
				T4 IRD	1.9	18	9	(9)
				T4S ORD	ND[b]	ND[b]		(10)
				T4S IRD	0.29	527	1820	(10)
				rT3 ORD	0.06	560	8730	(9)
				rT3S ORD	0.06	516	8600	(10)
				T3 IRD	6.2	36	6	(9)
				T3S IRD	4.6	1050	230	(11)

[a] The numbering of the amino acid residues corresponds to the position in human D1 [gi:3041700]
[b] Not detectable
[c] Ratio of the mean Vmax (pmol/min•mg protein) and Km (µM) values

Since a substantial fraction of serum T3 is derived from ORD of T4 by liver D1, at least in well studied subjects like rats and humans (12-15), the aberrant characteristics of cat D1 make the physiological role of D1 in cat liver an important issue. From the observation that selenium deficiency in kittens caused increased plasma T4 and decreased T3 levels (16) it might be concluded that also in cats, like observed before in rats (17-19), (liver) D1 provides a large part of circulating T3. This is in agreement with an earlier report, where, despite

differences in rT3 ORD, kinetic characteristics of T4 ORD of cat D1 were not different from rat and human D1 (1). Although we could not measure D2 activity in cat liver, an additional role for D2 in plasma T3 generation cannot be excluded.

Because rT3 is a poor substrate for cat D1, it would be conceivable to expect significantly higher serum rT3 levels in cat as compared to other species, like human or rat. Indeed, the rT3/T4 ratio for cats was elevated as compared to human in agreement with less efficient rT3 ORD by cat D1, whereas only slightly elevated total rT3 levels were observed. Therefore, alternative metabolic routes could well play an important role in rT3 degradation in the cat. Firstly, although T4 is the preferred substrate for D2, rT3 ORD is also catalyzed by D2. Although we neither were able to measure D2 activity in cat liver (low-Km and PTU insensitive ORD of T4) nor examined D2 expression in other feline tissues, this pathway could still be of importance in feline rT3 metabolism. In this respect it should be mentioned that, whereas only D1 activity is found in mammalian liver, D2 and D1 activity can be detected in teleost fish liver (20-24).

Secondly, the above-mentioned lower serum binding in cats and dogs also can account for rT3 levels being not as high as probably might be expected from the observation that rT3 is a poor substrate for D1 in both species. Since we only studied cat D1 we cannot exclude the possibility of lowered rT3 production by cat D3. Analogous to cat D1 it could well be that cat D3 also shows altered enzyme kinetics. To exclude this possibility we should have studied other feline tissues as well, like brain, because it would be conceivable to expect high expression levels of D3 in feline brain as observed in other species.

Furthermore, the physiological role of ORD of the biologically inactive rT3 probably is the recovery of the trace element iodine, which is then reutilized for *de novo* T4 synthesis in the thyroid gland. Sulfation of iodothyronines is an important metabolic pathway in thyroid hormone metabolism. Although water solubility of these lipophilic hormones is increased by sulfation and elimination in bile and/or urine accordingly facilitated (25-27), a more important purpose is that it leads to rapid degradation and irreversible inactivation of thyroid hormone by D1 deiodinase (28, 29). This can be deduced from earlier studies mainly performed in humans and rats, where sulfated iodothyronines like T3S and T4S are deiodinated by D1 40- to 200 fold faster, respectively, than the corresponding nonsulfated compounds (30, 31). As IRD of T3 and T4 as well as ORD of 3,3'-T2 are greatly facilitated by sulfate conjugation, whereas ORD of T4S is completely blocked (3, 8, 28-30, 32), it is obvious that sulfation leads to a rapid and irreversible inactivation of thyroid hormone by D1. Blocking of ORD is not a general phenomenon since ORD of rT3 by rat D1 is not affected by sulfation, whereas ORD

of 3,3'-T2 by rat D1 is accelerated 50-fold by sulfation of this compound (28, 29). Thus, deiodination of a number of iodothyronines by D1 is accelerated by sulfation of their phenol hydroxyl group. The facilitated deiodination of sulfated iodothyronines is an unique property of D1, because neither D2 nor D3 is capable of catalyzing the deiodination of these compounds (33, 34). This property of D1 was also observed for cat D1, in that deiodination was stimulated by prior sulfation of rT3 and T2.

Because rT3 ORD activity of cat D1 is very low, it seems plausible that in cat liver deiodination of rT3 occurs via prior sulfation of the substrate. Although we were able to measure T2 and rT3 sulfation activity in liver cytosol, we did not study the sulfation process in particular, i.e. we did not identify the responsible sulfotransferase(s) nor studied the kinetic characteristics. Since rT3 is a preferred substrate for sulfation by human SULT1E1 which is also expressed in liver, this could well be an alternative route for rT3 degradation. To test the hypothesis of successive sulfation and deiodination of rT3 in cats one could think of an in vivo experiment as described before for T3 metabolism in rats (35). This experiment should study the effects of PTU on plasma rT3S levels in cats after intravenous administration of radiolabeled rT3. In the control cats, i.e. cats that only receive rT3, rT3 enters the circulation and is sulfated in the liver and thereafter rT3S would be rapidly deiodinated by cat D1. In cats that receive PTU, sulfation of rT3 will continue whereas ORD of rT3S is inhibited. This results in an accumulation of rT3S in PTU-treated cats, whereas in the control cats' serum rT3S levels will be hardly detectable. Furthermore, to confirm the in vivo importance of cat D1 for ORD of rT3S, the effect of PTU on plasma rT3S levels should be studied after injection of rT3S. Whereas in the control group rT3S would be immediately metabolized by ORD activity of cat D1, in the PTU treated group an accumulation of rT3S is expected because of inhibition of this pathway.

Besides sulfation and D2 ORD activity, glucuronidation could also be an important pathway in rT3 metabolism in the cat. Conjugation of the phenol hydroxyl group with glucuronic acid by UDP-glucuronyltransferases (UGTs) renders the hormone more hydrophilic thereby facilitating the biliairy and fecal excretion. As was reported earlier in humans (36), the bilirubin and phenol UGTs UGT1A1 and UGT1A9 are responsible for the glucuronidation of T4 and rT3, with rT3 as the preferred substrate. These data concur with data from previous work performed in rats and cells expressing human UGTs, and additionally it was shown in the rat, that glucuronidation of T3 is performed by androsterone UGT (37, 38). Therefore, since rT3 is the preferred substrate for some UGTs and

glucuronidation has proven to be a metabolic pathway of importance in other species, this could well be an important pathway for rT3 metabolism in the cat.

As mentioned above, the kinetic characteristics of cat D1 resemble that of dog D1, but are quite different from that of rat, pig and human D1. Our data on cat D1 are in agreement with an earlier report on dog D1, besides the fact that these authors did not test rT3S as a substrate (3). Furthermore, it was reported earlier, that the relatively poor ORD of rT3 by dog D1, compared with human and rat D1, was related to differences in the sequence of amino acids 36-70 of the proteins (2). Therefore, it is not remarkable that we found changes in the amino acid sequence of cat D1 in this region as compared to the human and rat D1 amino acid sequence, namely N45R46 versus G45E46 (human) and G45Q46 (rat), L65Y66 versus F65F66 and a TGMTR (48-52) deletion (Table 1). On the other hand, dog D1 only differed in this region from cat D1 at amino acid position 46 (G vs. R), which residue is not well conserved.

It is remarkable that a combination of changes, i.e. the substitution of Phe for Leu at position 65, the insertion of five amino acids (TGMTR) and the substitution of N45R46 to G45E46 is necessary to obtain mutant cat D1 with rT3 ORD activities comparable to those of human and rat D1. As shown in Table 2, each substitution as such or a combination of 2 substitutions only shows minor improvements in rT3 ORD and even reduced catalytic efficiency with regard to rT3S ORD. Whereas insertion of TGMTR alone in cat D1 does not improve rT3 ORD and even markedly reduces rT3S ORD (CM5), the same insertion against a G45E46 and F65 background (CM14 and CM15), i.e. human background, shows major improvements in catalytic efficiency of ORD of both rT3 and rT3S. This is in agreement with an earlier report on dog D1, where insertion of TGMTR alone also did not improve rT3 ORD and inhibited rT3S ORD (3).

Table 2. Site-directed mutagenesis in canine and feline D1

Species	Reaction	Residue[a]			Km µM	Vmax pmol/min.mg	Vmax/Km[b]	Ref.
		45-46	48-52	65-66				
CAT wt	rT3 ORD	NR	-	LY	15	37	2/3	(7)
RAT wt		GQ	TGMTR	FF	0.2	38	221	
CM5		NR	TGMTR	LY	5	16	3/3	
CM1		NR	-	FF	14	78	6/7	
CM10		GE	-	LY	16	132	8/9	
CM11		GE	-	FF	6	129	22/39	
CM12		GE	-	FY	9	138	15/29	
CM14		GE	TGMTR	FF	0.7	65	93/126	
CM15		GE	TGMTR	FY	1.0	120	120/150	
DOG wt	rT3 ORD	NG	-	LY	27	92	3.4	(3)
HUM wt		GE	TGMTR	FF	0.5	141	288	
DM7		GE	TGMTR	FF	0.9	195	229	
DOG wt	rT3 ORD	NG	-	LY	9.0	17	1.9	(2)
HUM wt		GE	TGMTR	FF	0.4	74	185	
DM1		NG	TGMTR	LY	9.0			
DM4		GE	TGMTR	LY	3.8			
DM7		GE	TGMTR	FF	0.5	67	134	
CAT wt	rT3S ORD	NR	-	LY	0.7	57	81/81	(7)
RAT wt		GQ	TGMTR	FF	0.2	42	221	
CM5		NR	TGMTR	LY	5	42	8/9	
CM1		NR	-	FF	0.6	18	30/44	
CM10		GE	-	LY	3.4	165	49/52	
CM11		GE	-	FF	4	101	25/44	
CM12		GE	-	FY	1.9	79	42/81	
CM14		GE	TGMTR	FF	0.6	135	225/303	
CM15		GE	TGMTR	FY	0.9	220	244/300	
DOG wt	T3S IRD	NG	-	LY	6.9	1058	153	(3)
HUM wt		GE	TGMTR	FF	1.2	219	183	
DM7		GE	TGMTR	FF	4.8	431	85	
DOG wt	T2S ORD	NG	-	LY	7.4	239	32.3	(3)
HUM wt		GE	TGMTR	FF	0.6	57	104	
DM7		GE	TGMTR	FF	2.2	76.7	34.9	

[a] The numbering of the aminoacid residues corresponds to the position in human D1 [gi:3041700].

[b] As a measure for the catalytic efficiency the Vmax/Km ratio is presented either calculated from the measured values (3, 7) or after standardization for the amount of protein expressed (calculated values/values after standardization) (7).

However, it should be mentioned that in this study the insertion of TGMTR was only tested without the other substitutions, whereas the G45E46→N45R46 and F65F66→L65Y66 substitutions were always tested after TGMTR insertion (2, 3). Therefore, the conclusion of these authors that the TGMTR deletion does not play a critical structural role in D1 function was premature, because our data showed a positive effect on ORD of rT3 and rT3S of the TGMTR insertion in the presence of G45E46 and F65. Substitutions at all three positions are needed to obtain mutant cat D1 enzymes with catalytic efficiencies for rT3 ORD comparable to those of rat and human D1.

Insertion of TGMTR could either improve the positioning of F65 toward the inner-ring of rT3 and rT3S and/or the positioning of the outer-ring toward the catalytic center (Sec). From the fact that especially for rT3 the increased Vmax/Km ratio is mainly caused by a decrease of the Km value (3, 7) it might be concluded that improved interaction of F65 with rT3 is the major effect.

It has always been puzzling that liver microsomes can catalyze both IRD as well as ORD. That only a single enzyme is involved in both actions has been confirmed after cloning of D1 (9, 39-43). The observation of D1 performing both IRD as well as ORD could be explained by either different orientations of substrate binding within a single site, so that either the iodines of the inner or the outer ring are in close proximity to the Sec. Another possibility is that two overlapping binding sites connected with deiodination might exist, one site for ORD and one for IRD. On first sight, our data seem to support the two-substrate binding site model, that is, one binding site for substrates with two iodines in the inner ring and another site for substrates with two iodines in the outer ring. Also data in the dog, where identical amino acid substitutions led to improved rT3 and T2S ORD with unaffected IRD of T3S (3) support this model. Furthermore, this model could well explain the fact that T4 undergoes ORD as well as IRD, because at each ring T4, as the only iodothyronine, has two iodines available for interaction. Thus, two sites would exist for T4. However, the observation that sulfation of T4 gives a shift in deiodination preference argues against this model. Whereas IRD of T4 is strongly facilitated following sulfation, ORD of T4S is undetectable (29, 31). It is difficult to explain why the binding site connected with ORD would accept rT3S and T2S, but not T4S. This means that the model of one substrate-binding site is more likely with substrates binding in an orientation that either favors ORD or IRD. Crystallographic studies could contribute in the future to obtain more detailed insights in D1 structure-function relationship, although the problems encountered by overexpressing this membrane-integrated protein and rendering it in a soluble, active form should be overcome first.

It has been shown before that 3 histidine residues, i.e. His158, His174 and His185 (hD1 numbering), are conserved between human and rat D1. Multiple alignments with other mammalian D1 proteins show that only His158 and His 174 are well conserved within the D1 protein (2, 7, 39, 44). Systematic site-directed mutagenesis of these residues showed that His158 is critical for normal enzyme structure, whereas mutagenesis of His 174 to Gln or Asn causes a 20- to 100-fold increase in the Km for rT3 indicating that this residue is critically involved in hormone binding (45). Mutagenesis of His185 resulted in mutant D1 activity that was not different from wild type D1 activity. This is in line with our observations in cat D1, where residues 158 and 174 are conserved, but this is not the case for residue 185.

Deiodinases and thioredoxin-like superfamily

Although there is some structure-function information available, in particular for D1, our understanding of the catalytic mechanisms and three-dimensional conformation of deiodinases is limited. The integral membrane nature and the inefficient eukaryote-specific pathway for selenoprotein synthesis make it impossible to synthesize large quantities of soluble, catalytically active proteins for crystallization purposes. However, recently, in an attempt to gain further insights into the structures of these proteins, hydrophobic cluster analysis (HCA) was performed (46). Deiodinase sequences were used as queries in PSI-BLAST searches (47) (default values, nonredundant (NR, 1,027,609 sequences), and Swiss-Protein (SW 100,395 sequences) databases at NCBI). Just below the significance treshold value (expected E-value> 10^{-3}) similarities with various members of the thioredoxin (TRX) or TRX-like families were observed. Furthermore, two members of this family with known three-dimensional structure of the nonmembraneous part were added. This resulted in a model for the structure of the iodothyronine selenodeiodinases (46). Since similarities were observed between certain clusters of the different deiodinases and TRX-like proteins, it was proposed that the extramembrane portion of deiodinases belongs to the thioredoxin-fold superfamily, i.e. deiodinases, TRX-like proteins share the same ancestor.

The predicted structure of a single transmembrane domain connected to a globular domain containing the catalytic center, is compatible with the predicted data derived from protease protection assays, selective biotinylation, and immunofluorescence cytochemistry (48-50). However, it is unclear if TRX is a suitable template for the deiodinases model. The homology between TRX and the deiodinases, as mentioned above, is rather low and also TRX is not able to bind TH. Furthermore, unlike D1-3, TRX is not a membrane protein. Since this model did not include docking of the substrate, no information was obtained to support the

one-substrate or two-substrate binding site model for D1-catalyzed IRD and ORD as discussed above.

D1 and PTU sensitivity

Although this model could explain a number of results from previous mutagenesis analyses the explanation for some observations remain obscure. With regard to inhibitors D1 protein is strongly inhibited by PTU (IC_{50} = 5 μM), GTG (IC_{50} = 0.05 μM), and IAc (IC_{50} = 2 μM). The reactive Sec residue within the active center of the enzyme is thought to be the target for these inhibitors (51-53), where IAc and GTG react with this residue in its reduced (SEH) form and PTU with the selenenyl iodide (SeI) intermediate (54, 55). Although D2 also contains a Sec residue in the catalytic center it is much less sensitive to inhibition by PTU (IC_{50} > 1000 μM), GTG (IC_{50} = 1 μM) and IAc (IC_{50} = 1 μM). Since all PTU-sensitive deiodinases, i.e. human, rat, mouse, dog, chicken, cat (2, 7, 39, 44, 56, 57) and porcine D1 (this thesis), have Ser at position 128, and all PTU insensitive deiodinases, i.e. all D2s (58-61), all D3s (62-65), tilapia D1 (66), killifish (21) and trout D1 (Genbank) a Pro at the equivalent position it was postulated that this was a significant residue for PTU sensitivity. In an attempt to restore PTU sensitivity the Pro128Ser mutant of tilapia was prepared. The mutant enzyme showed strongly decreased ORD and somewhat increased IRD activity, but was still PTU insensitive (66). In an approach to elucidate important amino acid residues for PTU sensitivity the human D2 Pro135Ser mutant was prepared. This yielded a mutant D2 enzyme with ping-pong kinetics and an increase in Km for T4 by two orders of magnitude, which became PTU-sensitive in a competitive manner with DTT. The same was observed with the human D3 Pro146Ser mutant, whereas the mutant D1 Ser128Pro enzyme had the same Km and ping-pong kinetics but became PTU-resistant, suggesting that there was no longer an accessible E-SeI intermediate (46). The lack of PTU inhibition of the tilapia D1 Pro128Ser mutant indicates that also other amino acid changes play a role.

The data on tilapia D1 suggested a K_{cat} value lower than for mammalian D1s, which could be explained by a lower reactivity of Sec in tilapia D1 (66). Because PTU reacts with an enzyme SeI group, the rate of formation of this intermediate also determines susceptibility to PTU inhibition. In this regard it is interesting to mention that the basic Lys and Arg at position 11 and 12 of mammalian D1, which are important for membrane insertion (49), are lacking in teleost fish tilapia and killifish D1 (21, 66).

To test the hypothesis that the Ser residue at position 128 in D1 is essential for PTU sensitivity a less drastic change in amino acid composition at this position is favorable.

Substitution of a Pro for a Ser induces more changes than just another amino acid at position 128. Because of these confounding factors this mutation therefore is not appropriate for studying the role of residue 128 in PTU sensitivity. Therefore, a study on PTU sensitivity should be performed by a more subtle mutation such as human D1 Ser128Ala. This could help to give more insight in the process of PTU sensitivity.

Besides a PTU insensitive D1, teleost fish differ in that the liver of rainbow trout and killifish expresses both D1 and D2. Furthermore, tilapia liver expresses only D2, and D1 activity was reported in tilapia liver only after administration of methimazole (20, 22-24). Furthermore, T4 ORD seemed to be performed solely by D2, whereas rT3 ORD was performed by both D1 and D2 (20). This has physiological significance with regard to thyroid hormone peripheral parameters. Fish exhibit low circulating levels of T4 and high levels of T3, resulting in the higher T3/T4 ratio characteristic of teleostean species. This fits well with an important role for D2 in systemic supply of T3 unlike mammals, where circulating T3 is supposed to be generated mainly by T4 ORD by liver D1. Furthermore, it was reported that in trout liver no evidence was found for thyroid hormone sulfate deiodination (67), which is a key property of rat D1. This was concluded from the observation that deiodination of T4S, T3S and rT3S was negligible, where simultaneously tested rat liver microsomes rapidly deiodinated these compounds. Furthermore, rT3 ORD was not competitively inhibited by T4S, T3S, or rT3S. However, the thyroid hormone sulfate concentrations used were not higher than 100 nM and with regard to the Km of trout D1 for rT3 (200 nM) this concentration is too low to expect inhibition and subsequently draw any conclusions. A report on tilapia D1 showed IRD of T3S, showing substrate specificity for rT3>>T4>T3S>T3 (66). Comparative data on catalytic efficiencies (Vmax/Km ratios) for T3, T4, rT3 and T2 and their respective sulfated counterparts should be tested in tilapia D1 to answer the question, whether sulfation of thyroid hormone analogs enhances D1 deiodination as observed for mammalian D1, or whether sulfation decreases deiodination as was reported for trout D1. To confirm that the observation in trout is an intrinsic property of teleost fish D1 the same study should be performed for killifish D1.

Facilitated deiodination of sulfated compounds

Toyoda et al. (3) observed that ORD of substrates with one iodine on the inner ring (rT3 and T2S, respectively) is favored by Phe at position 65, whereas the deiodination of substrates with two iodines on the inner ring is independent of the occupation of position 65 by Phe. In our study on cat D1 (this thesis), rT3 ORD was also improved by the substitution of Phe for

Leu65, thereby confirming the observation of Toyoda and coworkers. Although not tested in our study, a Tyr residue at position 65 could probably perform the same role as Phe in ORD of rT3. This could be concluded from an earlier report on killifish D1 (Tyr65Gly66), where Km for rT3 was in the same range as observed for human and rat. However, kinetic data were incomplete because Vmax, an important denominator in the determination of catalytic efficiency, was not measured (21). Moreover, killifish D1 contains VTMTQ at postions 48-52 and GE at positions 45 and 46. Our study showed high catalytic efficiency for rT3S in cat D1 (Leu65Tyr66) with a decline in efficiency when residue 66 was substituted with Leu. It seems that where residue 65 (Phe or Tyr) is important in ORD of rT3, residue 66 (Phe or Tyr) is important for ORD of rT3S. However, the combination of two Tyr residues at positions 65 and 66 has a negative effect on both rT3 and rT3S ORD, although this has not been tested in the presence of both 48TGMTR52 and G45E46.

The high degree of homology between the species with regard to residue 65 (all known D1s have a Phe65, except cat (Leu), dog (Leu) and killifish (Tyr) D1, and all D2 and D3 enzymes have another residue at this position) fits well with rT3 as preferred substrate for all D1s (except for dog and cat D1). A significantly lower degree of homology for amino acid residue 66 could explain the differences observed between deiodination of sulfated iodothyronines by mammalian and teleost fish D1. Therefore, kinetic studies with sulfated and unsulfated substrates on wild-type and mutant fish D1 would be very informative in answering whether residue 65 is important in the deiodination of unsulfated hormones, and residue 66 in the deiodination of sulfated analogs. Testing amphibian D1 could derive additional information. Because amphibians exhibit Phe65Phe66 it is conceivable that sulfated as well as unsulfated iodothyronines are good substrates for amphibian D1. Moreover, in killifish D1 the Tyr65Tyr66 mutant versus wt enzyme could be studied, to assess whether this combination has a negative effect on both rT3 as well as rT3S ORD.

The facilitated deiodination of sulfated iodothyronines by mammalian D1 could be explained by the negatively charged sulfate group interacting with a positively charged side group of a basic amino acid residue, Arg or Lys, thereby stabilizing the interaction with D1. Since deiodination of sulfated compounds is not facilitated by D2 and D3, and assuming a similar location of the substrate-binding domain in the different deiodinases, the residues involved should differ at equivalent positions between D1 *versus* D2 and D3, respectively. This is the case for residues 76-81 (hD1 numbering), where the net charge in mammalian D1 is positive, while the net charge is neutral for D3 or even negative for D2 (Table 3). This seems to be in accordance with the data on deiodination of sulfated compounds, but the

ultimate proof would be site-directed mutagenesis on residue 76, 78 and 81 of hD1. Moreover, exploring the shrew would be very informative as well, since shrew D1 differs from all other mammalian D1s in that the net charge of shrew D1 is only +1 (Table 3), but no data on deiodination of sulfated iodothyronines by shrew D1 are yet available. Furthermore, the same difference in net charge was observed between mammalian D1 *versus* amphibian and teleost fish D1, D2 and D3, respectively. Whereas in trout liver no evidence was found for deiodination of sulfated compounds (67) in tilapia D1 IRD of T3S was described (66). Therefore, again, comparative data on catalytic efficiencies for T3, T4, rT3 and T2 and their respective sulfated counterparts are needed before any conclusions can be drawn whether sulfation can also facilitate deiodination by teleostean and amphibian D1. Nevertheless, charge differences between mammalian *versus* teleostean and amphibian D1 are present as observed between mammalian D1 *versus* D2 and D3. Therefore, it could well be, that, analogous to mammalian D2 and D3 and regardless of the favorable situation at position 65/66, sulfation does not facilitate deiodination by teleostean and amphibian D1.

In the linear context the above-mentioned residues lie relatively close to residue 65 and 66. Since our knowledge about three-dimensional structure of deiodinases is limited it cannot be ruled out that other, yet unknown, residues are equally or even more important because they are closer to position 65-66 than one would expect from linear structure alone. What can be told of residue 76-81 is, that in the model for the structure of the iodothyronine deiodinases (46) these residues are located in an α-helix structure in the so called N-linker region, a region linked to the transmembrane region via a hinge. The α-helix comprises amino acid residue 59-85, i.e. according to this model residues 65 and 66 and residues 76, 78 and 81 lie in the same α-helix.

Table 3. The amino acid residues at position 76-81 in human D1 and at equivalent positions in other D1, D2 and D3 proteins and the net charge of these residues

Species	D1		D2		D3	
	Residues	Charge	Residues	Charge	Residues	Charge
Human	KVRWQR	+3	KSFLLD	0	HGQKLD	0
Pig	KVRWQR	+3	KSFLLD	0	HGQKLD	0
Rat	KVRWQR	+3	NSFLLD	-1	HGQKLD	0
Mouse (mm)	KVRWQR	+3	NSFLLD	-1	HGQKLD	0
Shrew (sm)	KVNWQQ	+1	Unknown		Unknown	
Dog	KVQWQR	+2	KSFLLD	0	HGQKLD	0
Cat	KVRWQR	+3	Unknown		Unknown	
Chicken	KVKWRR	+4	NSFLLD	-1	HGQKLD	0
Xenopus	EIMWMR	0	NSFLLD	-1	HGQKLD	0
Rana	Unknown		NSFLLD	-1	YGQKLD	0
Tilapia	SHMWLS	0			HGQKLD	0
Killyfish	QTMWLS	0	NSFLLD	-1	Unknown	
Trout	GHMWTN	0	NSFLLD	-1	Unknown	
Zebrafish	FVNWCS	0	NSFLLD	-1	YGQKLD	0
Pufferfish	DNMWLS	-1	NSFLLD	-1	YGQKLD*	0
					HGHKLD*	0

*) two D3 subtypes

All together, involvement of positively charged residues at positions 76-81 in facilitated deiodination of sulfated iodothyronines may be deduced from the observation, that this facilitation is absent in mammalian D2 and D3, where the net charge is neutral or even negative at equivalent positions. A definite proof would be the loss of facilitated deiodination of sulfated compounds after diminishing the positive charge by site-directed mutagenesis at positions 76-81 in mammalian D1. Furthermore, testing shrew D1 kinetics would be informative as well. Further tests to confirm the hypothesis of involvement of positively charged residues at positions 76-81 in the facilitation of deiodination of sulfated compounds would be kinetic studies on deiodination of unsulfated iodothyronines as well as their sulfated counterparts by amphibian and teleost fish D1.

What could be the physiological explanation for the negligible deiodination of sulfated thyroid hormones in teleost fish? Fish probably do not rely on iodine recycling, because they continually take up ambient iodine across the gills thereby maintaining plasma iodine levels even in freshwater (68, 69). Thus, fish, unlike mammals, do not depend exclusively on dietary iodine sources and as a consequence there may have been no strong selective advantage for fish to evolve an enzyme comparable to mammalian D1, which facilitates deiodination of

sulfated compounds to preserve iodine. Thyroid hormone is largely secreted in teleost bile as glucuronide or sulfate conjugates (67, 68).

There is a high degree of homology between the amino acid sequences of porcine and human D1, and kinetic parameters of porcine D1 are almost identical to those of human and rat D1. However, they differ with respect to deiodination of T2S. Whereas no or a little IRD was measured of T2S by rat and human D1, porcine ORD and IRD were measured in equal amounts in porcine D1. It is for the first time that IRD of T2S is reported. T4S IRD has only been reported for rat D1 (10). It is interesting to test whether porcine D1 is able to perform both T4S IRD and ORD, and, if so, amino acid composition of rat and porcine D1 should be compared. T4S and T2S differ from other sulfated iodothyronines in that there is a balanced amount of iodide in the inner- and outer-ring, i.e. 4 versus 2, where there is imbalanced situation with respect to this in other iodothyronines. Together with the negatively charged sulfate-group this could strengthen binding of the substrate in a particular orientation, bringing either the outer- or inner-ring in close proximity to the catalytic center. This could explain the difference between T4 and T2 (both ORD and IRD) and their sulfated counterparts (T4S only IRD and T2S only ORD, respectively). In porcine D1 there could be a counteracting mechanism like charged amino acid residues that interfere, hampering strong substrate binding and allowing binding in different orientations which allows for IRD of T2S and perhaps also ORD of T4S. Interesting residues, amongst others, are the residues we described in feline D1 (45-46, 48-52). Whereas rat D1 has G45Q46 and TGMTR, pig D1 has S45Q46 and TGMAK. Furthermore, at position 158, 174 and 185 a His residue was present in porcine D1 as was observed for human D1. Together with D1 sequence information from other species, like cat as mentioned earlier, this confirms the importance of a His at position 158 and 174. Cys124 and Cys194 are also conserved in pig D1.

The porcine deiodinase sequences, as compared to other mammalian sequences, were most homologous to hD1-3. We aligned these sequences with other mammalian D1-3 sequences to detect homologous and deiodinase-specific residues (Fig.1 chapter 4). A rational approach to study the structure-activity relationship of deiodinases is to modify amino acid residues which are strongly conserved within but differ between deiodinase subtypes. The substitution of these residues may result in changes in deiodinase specificity, whereas substitution of residues conserved in all deiodinase subtypes are expected to have deleterious effects on enzyme function in general.

A number of residues located in the putative deiodinase active center (amino acid residue 115-156 in the model of Callebaut et al. (46)) are well conserved throughout the three

deiodinases. Besides Sec126 these residues are Phe121, Ser123, Phe129, Glu156 (D1 numbering). This holds true for the deiodinase-specific insertion (amino acid residue 157-182 in the same model) at position His158, Ser160, Trp163, and His174 (D1 numbering). As mentioned before, since these positions are well conserved, it is conceivable to expect drastic effects when these positions are mutated. Indeed, this has been shown earlier by site-directed mutagenesis on residue Sec126, Glu156, His158, and Trp163 in hD1 and equivalent positions in hD2 (Introduction, Table 4A and B). Mutations on the first 4 residues were deleterious on enzyme activity in D1 and D2, while only the first 2 were tested in hD3 but with identical results. Mutagenesis of these well-conserved residues results in complete loss of activity, suggesting a role in either protein conformation or catalysis. Whereas two essential His residues are present in D1, i.e. His158 and His 174, this is only partially true for D2. Although equal substitution of the residue equivalent to His158 in D2 showed the same deleterious effect, the same substitution at position equivalent to His174, however, had no effect on enzyme kinetics in D2 (46). Thus, whereas two essential His are present in D1, there is only one for D2. An essential role of His 174 in maintaining the reducing environment for the Se active center (13) seems unlikely, since substitution of this residue has different effects on D1 and D2 enzyme kinetics. No information is available about the importance of His176 and His193 in hD3, positions corresponding to His158 and His174 in hD1, respectively. Mutational analysis should indicate if both histidine residues in D3 are essential or if this is a specific feature for D1.

Another reasonable approach is to identify and characterize deiodinases with aberrant enzyme characteristics. This helps in structure-function research because differences in residues can be directly linked to functionality. This we have shown for cat D1, where aberrant D1 kinetics could be related to altered amino acid residues. In future studies on non-mammalian deiodinases, such as the teleost fish and amphibian enzymes, an identical approach as described here for cat and porcine D1 should be followed.

In conclusion, structure-activity relationships can be studied in different fashions. Identification of deiodinase type-specific residues by multiple alignments of the three deiodinases of different species followed by site-directed mutagenesis can be performed. However, this method yields a lot of virtual sites for site-directed mutagenesis. Therefore, one should combine these data with additional information about functionally important residues. Furthermore, deiodinases with aberrant enzyme kinetics can be explored. Unfortunately, naturally occurring mutations in the different deiodinases have not been reported so far.

D1 splice variants

In chapter 3 we describe a study on alternative splicing of hD1. Splicing is not a random process and since intronic GT and AG at the 5' and 3' splice junctions are highly conserved, i.e. 99%, these sites seem to be consensus splice sites (70). Splicing out of a whole exon from human D1 mRNA was observed as well as splicing within an exon. In most variants GT and AG were involved as donor and acceptor sites, respectively. Whenever this GT-AG consensus was followed in these rare variants we considered them to be real splice variants, whereas some variants that did not obey this consensus were not regarded as such.

Variant D1 mRNA was found in liver, kidney and thyroid, but since variant human D1 mRNAs were also identified in human testis (Genbank: gi15252261 and gi15253482), the occurrence of splice variants is not limited to typical D1 tissues. We concluded that alternative splicing seems to be widespread and there was no correlation observed in fetal liver between gestational ages and splicing events. Although this was the first report on hD1, alternative splicing in coding as well as non-coding region of hD2 has been described earlier (71-73). Whether these hD1 variants also exist at the protein level remains unclear since the epitope of our D1 antiserum was at the C-terminus and therefore it would only detect a limited number of D1 variants (Chapter 3, Table 1). Therefore, to test this hypothesis D1 antibodies should be raised against different epitopes.

Provided that the variants exist at the protein level there are 2 interesting variants. The most frequent variant we cloned was hD1e and this sequence was also found 3 times in Genbank (EST-clone) (Chapter 3, Table 1). hD1e preserved Sec in the virtual protein, a prerequisite for D1 activity. This feature was shared with another variant, hD1b, whereas all other variants did not preserve Sec. Besides Sec, hD1e contains the essential amino acids between residues 30-70 that account for the difference in Km for rT3 ORD between dog and cat vs. human (2, 3, 7). However, hD1e lacks one of the essential active site His, i.e. His174, and this could have consequences for enzyme activity since site-directed mutagenesis in the rat at this position resulted in a significant increase in Km for rT3 ORD (45). Variant hD1b was observed less frequently but also identified in Genbank. This variant lacks residues 49-113, i.e. an important part of residues 30-70, critical for D1 function. All in all, in liver, thyroid and kidney human hD1 mRNA undergoes alternative splicing. Although regulation of D1 expression by T3 is generally considered to occur at the transcriptional level (74-79), effects of T3 on D1 splicing cannot be excluded and remain to be investigated. D1 antibodies should be raised against different epitopes to identify each variant at the protein level. In the

context of structure-function relationship transfection and activity studies should be performed. Since the Sec residue is a prerequisite for deiodinase activity, it is difficult to predict the value of the virtual proteins without Sec. However, since residue 30-70 contain amino acids that are important in substrate interaction (2, 3, 7), it is conceivable that the variants where Sec is lacking but residues 30-70 are still present (hD1c, hD1d, hD1f, hD1l, respectively) fulfill blocking activity by binding the substrate and thereby hampering deiodination by wild-type D1.

II Pathophysiological importance and regulation

Thyroid state and deiodinases

In our study where we tested the suitability of the pig as an animal model for human thyroid hormone metabolism regulation of pD1-3 by thyroid state, amongst others, was studied. In the thyrotoxic state pD1 activity in liver and kidney was increased. This is in line with earlier reports on rat D1 (80). Identification of TREs in the promotor region of the human D1 gene (74, 79) indicate that regulation of D1 in humans is exerted at the level of gene transcription. Whether this holds true for porcine D1 has to be investigated. D3 was identified in porcine brain and showed the same positive regulation by thyroid state as was reported before for rat brain (81, 82). However, the mechanism behind this thyroid hormone dependent regulation of D3 expression remains to be established.

Probably the most remarkable finding was the induction of D2 activity in both thyroid and skeletal muscle in methimazole treated pigs. Although the TSH assay we used showed only minor cross-reactivity with porcine TSH we still were able to detect TSH under these circumstances, indicative for highly elevated TSH levels. A number of conditions in humans have been described where thyroidal D2 activities were significantly increased, i.e. in patients with Graves' disease or hyperfunctioning adenomas, and in some patients with large or widely metastatic follicular carcinoma (83-85). These increased activities are of significance in that they increase serum free T3 levels (86). The same induction of D2 activity was observed in porcine skeletal muscle. D2 mRNA and activity were identified earlier in human skeletal muscle (59, 60). In the thyroid the upregulation of D2 is performed by TSH through the cAMP protein kinase A pathway (83, 84, 87), whereas the role of TSH is performed by TSH receptor antibodies in Graves' disease (88). In skeletal muscle, however, the increased D2 activity is explained in part by a reduction of T4-induced D2 proteolysis. Intrathyroidal T4 to T3 conversion by D2 may contribute significantly to the relative increase in thyroidal T3

production by D2 in conditions like Graves' disease, hyperfunctioning adenomas and large or widely metastatic follicular carcinomas. In iodine deficiency upregulation of thyroidal D2 activity could also be an important tool for the thyroid to improve iodine economy to maintain adequate iodine levels. The expression of D2 activity in porcine thyroid and skeletal muscle raises the question whether the induction of D2 in hypothyroidism can be a mechanism to counteract the low serum T3 levels.

D3 and heart failure

Heart failure is the clinical syndrome that results from the inability of the heart to pump sufficient blood to meet the metabolic requirements of the body or to do so only at an elevated ventricular filling pressure. Symptoms of heart failure are usually impaired exercise tolerance and symptoms related to fluid overload (89). In the heart, the transition from the fetal to the adult phenotype is generally dependent on the perinatal rise of systemic thyroid hormone (TH) levels (90). The expression of key cardiac proteins remains dependent on TH in the adult as evidenced by the profound changes in cardiac performance in the transition from hypo- to hyperthyroidism. Most notably, T3 stimulates the expression of the Ca^{2+}-pump of the sarcoplasmic reticulum (SR Ca^{2+}-ATPase, SERCA2), while reducing the expression of both the SERCA2-inhibitory protein phospholamban (PL) and the plasma membrane Na^{+}-Ca^{2+}-exchanger (NCX). This results in increased relaxation rates and increased Ca^{2+}-filling of the SR. The release of Ca^{2+} is also facilitated, through enhanced expression of the SR Ca^{2+}-release channel, the ryanodine receptor (RYR). The speed of contraction is increased by stimulation of expression of the faster myosin heavy chain (MHCα), while the slower isoform is repressed (MHCβ). These nuclear mediated actions are responsible for the positive inotropic and lusitropic effects. Together with the positive chronotropic effect of T3 the hemodynamic load placed on the heart is increased and this is the major stimulus for cardiomyocyte growth. The effects on cardiac growth and contractility result in a rise in cardiac output adapted to the higher metabolic demands and this form of hypertrophy is generally referred to as physiological (90).

In contrast, the form of hypertrophy we studied was caused by chronic pressure overload. This and also volume overload, due to hypertension, valvular dysfunction or loss of viable tissue after infarction, yields a form of hypertrophy leading to decompensation and failure (pathological hypertrophy). Re-direction of key cardiac ventricular proteins as mentioned above is for many enzymes the opposite of the changes induced by T3 (Table 4), i.e. a decrease in the MHCα/β ratio, repression of SERCA2 and RYR, and upregulation of

NCX and PL. This results in prolonged Ca^{2+}-transients, increased diastolic Ca^{2+}-concentration and reduced peak Ca^{2+}-concentration. This impairment in Ca^{2+} handling together with the changes in expression of contractile proteins is thought to be a primary cause of the systolic and diastolic dysfunction observed in pathological hypertrophy and failure in human and rat. Because of the similarities in the pattern of gene expression in the diseased heart and the hypothyroid state, a role for TH in the development of cardiac hypertrophy or heart failure has been suggested (91-93), i.e. impairment of TH signaling has been suggested as a factor in re-directing gene expression. This could be exerted at multiple levels, by diminished expression of nuclear TH receptors (TR) (92) and by diminished transport of TH into the cardiomyocyte, since there is evidence for existence of a transport protein for T3 as well as the involvement of fatty acids in TH transport into the cardiomyocyte (94, 95). Our hypothesis was that altered conversion or degradation of TH in the cardiomyocyte results in reduced biological activity in pathological hypertrophy and that this is important in the pathophysiology and maintenance of pathological cardiac hypertrophy and failure.

Table 4. Cardiac gene expression in heart failure and in hypo- and hyperthyroidism

	Heart failure	- T3	+ T3
SERCA	↓	↓	↑
RYR	↓	↓	↑
NCX	↑	↑	↓
MHCα	↓	↓	↑
MHCβ	↑	↑	↓

Although NTI was shown in the cardiac failure group, plasma TH levels in the hypertrophy group were not different from control values; yet, expression levels of T3-responsive genes in the hypertrophy group differed significantly from that in the control group. It seems that the development of pathological hypertrophy is largely independent of changes in plasma TH levels. This was also suggested by previous work. Normalizing plasma levels in a rat model of LV hypertrophy following myocardial infarction restored the mRNA level of MHCβ, whereas those of MHCα and SERCA2a were unaffected (96). Furthermore, in a rat model of pressure-overload induced LV hypertrophy, a nearly complete shift in MHC isoform expression and a 50% reduction of SERCA2a mRNA was observed without any significant changes in plasma TH levels (92).

With regard to local thyroid hormone metabolism activities of D1-D3 were measured. Whereas D1 activity was present at very low levels, D2 activity could not be detected. This is in agreement with earlier studies. In human heart D2 mRNA without D2 activity was measured (60, 97). In porcine and rat heart, we were not able to detect any measurable degree of D2 activity, even after rendering the pigs deeply hypothyroid. In contrast, in the typical D2-expressing tissues like pituitary and cerebrum, but also in muscle and thyroid, the D2 response was as expected, i.e. a big increase in D2 activity. Furthermore, exploration of the *Pax-8* knockout mouse, characterized by severe congenital hypothyroidism, also did not yield any D2 activity in the heart. Therefore, local conversion of T4 to T3 does not occur to any measurable degree in the cardiomyocyte. However, in contrast with our findings and those of others it was reported recently that D2 activity was measured in methimazole-treated rats and mice (98). Because only I^- production was measured and no data on T3 production were available, it cannot be ruled out that a nonenzymatic process is responsible for the I^- release. Since these authors find release of I^- in mouse as well as rat this could be a general problem of a nonspecific, nonenzymatic deiodination.

Whereas circulating TH and local T3 production are of minor importance, local degradation of T3 by D3 plays an important role in cardiac pathological hypertrophy and failure. We showed ventricle-specific induction of D3; this induction is of consequence for local T3 levels, in that local T3 in the failing heart is decreased. D3 is expressed in the fetal stage of different tissues and recently significant activity was detected in fetal human heart, whereas little activity was shown in adult human heart (R. Hume and T.J. Visser, unpublished observations). The same observation was made for human fetal liver (99). D3 is considered to be an oncofetal gene because it was also found in hepatocarcinoma cells (100) and is up-regulated by growth factors (101). This is an interesting aspect of D3 regulation since the re-expression of various fetal genes is characteristic for cardiac hypertrophy (91, 102). Therefore, enhanced D3-expression may be part of such a fetal gene program that needs to be activated to allow cell growth (103).

In continuation of this study a time series analysis of cardiac gene expression, deiodinase activities, and tissue TH levels in RV and LV was performed during the development of either compensatory hypertrophy or CHF to assess the precise role of induction of D3 activity in de pathogenesis of CHF. These studies were described in chapter 6. RV-specific massive induction of D3 activity was detected in CHF, coinciding with the start of development of heart failure. Furthermore, a RV-specific decrease in the ratio of tissue T3/T4 was observed in CHF hearts only. This cannot be attributed to low serum T3

levels, since the decrease is RV specific. Therefore, from this study we concluded firstly that induction of D3 activity contributes to the local hypothyroid state in the failing heart, thereby contributing to the development of heart failure. Whereas this mechanism is possibly part of the onset of the re-expression of a fetal gene program, its presence only in CHF hearts suggests a late activation only in response to a sustained pathological stimulus or an additional, yet unknown, stimulus. Secondly, altered TH metabolism is a contributor to the suppression of SERCA2a gene expression in severe heart failure. Since progressive detoriation of Ca2+ homeostasis is thought to be critical in the transition from compensatory hypertrophy to heart failure and the importance of SERCA2a in myocardial function has been proven by several studies, where prevention of the decrease in SERCA2a improved myocardial function, we propose that prevention of the events resulting in a decrease in local TH levels could be beneficial to prevent myocardial impairment in vivo. III) Additionally, shifts in gene expression of key cardiac proteins as observed in CHF can occur in a TH-independent fashion. This was described previously in the same animal model in regard to alterations in the β-adrenoreceptor-adenylate cyclase system. Neuro-endocrine overstimulation was postulated as underlying mechanism.

Furthermore, since there is evidence for existence of a T3 transport protein it is obvious that inhibition of T3 transport also could result in local hypothyroidism (94). In starvation and NTI, T4 uptake in the liver is decreased. This is explained by ATP depletion and increased concentrations of circulating inhibitors, such as nonesterified fatty acids and bilirubin (104). However, although there is a NTI in CHF, the effect of inhibitors on T3 transport in the heart remains to be resolved.

Our data do not negate the importance of possible defects in the signal transduction system in the pathogenesis of pathological hypertrophy, since down-regulation of TR in chronic LV-pressure overload was reported (92). More recently, the importance of regulation at TR level in the process of cardiac hypertrophy was again reported, but now in the context of exercise training-induced hypertrophy (105). Whereas pathological hypertrophy eventually leads to CHF, the form of hypertrophy as observed in exercise training is beneficial for cardiac function. However, the molecular mechanisms behind the improvement in cardiac function by exercise training are unclear. The beneficial effects of exercise training on cardiac function was studied in the aged rat (105). Exercise training improved the aging-induced decrease in myocardial contraction and relaxation and this is a beneficial adaptation in the aged heart. It was demonstrated at the molecular level that exercise training improved the aging-induced decrease in TRα1 and β1 and RXRβ protein expression and activity of

myocardial TR DNA binding to TRE. This resulted in improved protein expression of SERCA2a and MHCα. These results suggested that exercise training improved the age-induced downregulation of TR signaling in the heart. The authors proposed that regulation at the molecular level mediates TR signaling in the heart and partly contributes to the mechanism of the beneficial adaptive response to exercise training in old age. In conclusion, these data and those on pathological hypertrophy showing regulation of the same key cardiac proteins in an opposite direction (92) show that the TR is an important target for both physiological and pathological hypertrophy. This fits well with our data on induction of D3 activity. Since D3 was only present in CHF-hearts this suggested a late activation. Therefore, we regard induction of D3 activity as a key factor contributing to a more pronounced local hypothyroid state in the failing heart, thereby contributing to the development of heart failure. Initial events however would rather be located at the TR level.

New therapeutic options in CHF

Our data and those of others could well lead to the development of alternative therapeutic approaches in the treatment of CHF. The important role of TR in the development of hypertrophy makes a thyromimetic with selective TRα1 binding activity targeting the cardiomyocyte an interesting compound. As mentioned before, cardiac gene expression is mediated primarily by the TRα1 isoform, and TRα1 mRNA expression is significantly decreased in CHF (92). Therefore, this compound would have a thyromimetic effect on the cardiomyocyte lacking the unwanted effects on oxygen metabolism mediated by TRβ, thus allowing for chronic treatment (106).

Another interesting analogue is 3,5-diiodothyropropionic acid (DITPA). Compared with the biological active compound T3 it lacks an iodine atom in the outer ring and a NH_2 at the middle of the alanine side chain. Because of its possible preferential effect on myocardial contractility, this analog was already used, with the purpose of improving heart function after infarction, but this was mainly tested in animal models (107-110). Recently, in a pilot study, DITPA administered to patients with moderately severe heart failure increased cardiac index and decreased systemic vascular resistance. Total serum cholesterol and triglycerides were also decreased significantly (111, 112). Furthermore, in a post-infarction model DITPA prevented the decrease in SERCA2a, PLB and RYR protein post-infarction, without measurable changes in mRNA levels (110). DITPA favorably alters the stoichiometry between the calcium pump of the SR and its inhibitor PLB and has positive ino- and lusitropic effects without affecting heart rate (113). Heart rate is an important determinant of O_2-

consumption. This favors DITPA above TH. Because DITPA showed thyromimetic activity on key cardiac genes as described above it is conceivable that it is taken up by cardiomyocytes. An indirect proof for this is, that inhibition of plasma membrane uptake of T3 is observed in the presence of DITPA (114). Furthermore, it has been shown that DITPA binds to bacterially expressed thyroid hormone receptors (108). Since DITPA can exert its effects in heart failure, i.e. in presence of high D3 activity, it is tempting to speculate, that DITPA is not deiodinated as efficiently as T3 by D3. This could explain how DITPA can exert its effect within an environment of high D3 activity. Since it is effective in CHF it could well be that the activity of myocardial TR DNA binding in the MHCα and SERCA2a genes is higher with DITPA as with T3, or it could be equal to T3, but where T3 is quickly deiodinated by D3 DITPA is more resistant to D3 degradation.

A D3-specific inhibitor targeting the cardiomyocyte would also be an interesting analogue. This would result in restoration of local thyroid hormone concentrations in the cardiomyocyte without unwanted side effects on other D3-expressing tissues, like the brain. One could think of using an inhibitor that is transported by the MCT8 thyroid hormone specific transporter (115), which is present in heart cells. Although MCT8 is also present in neurons in the brain, this could be less of a problem, since this inhibitor may not be able to pass barriers on its way to these neurons, including the blood-brain barrier and astrocytes. Another possibility is the use of an inhibitor that is transported by the FAT fatty acid transporter, since recently transport of T3 and other iodothyronines by this transporter was shown (95). Since 70% of the cardiac energy demand is met by fatty acids and 30% by oxidation of carbohydrates (95), most of the inhibitor would end up in the heart. However, it could well be that transporters are downregulated in the failing heart analogous to the downregulation at receptor level (92). If this is true targeting of the inhibitor to the heart via thyroid hormone transporters is no option.

There is a growing number of cardiac disease states in which thyroid hormone metabolism is altered leading to a fall in serum T3. Within 48 h after acute myocardial infarction (116) or within 6-24 h after cardiac surgery requiring cardiopulmonary bypass in adults and children (117, 118) serum T3 declines . In patients with CHF, it has been observed that as many as 30% have low T3 levels that correlate with the severity of the clinical assessment of heart failure (119, 120). These observations led to the suggestion that the fall in serum T3 may adversely effect cardiac function (121), and similar to hypothyroidism (119, 122, 123) benefit from hormone replacement. It has been suggested to give replacement doses of T3 to restore serum levels (121, 124). However, our results on CHF suggest that T3

substitution in CHF is not beneficial. The substituted T3 will be degraded by locally induced D3 activity in the failing heart. Besides, because of the adverse increase of cardiac work via stimulation of the metabolic rate, T3 is not the treatment of choice in CHF in the first place.

Finally, alternative strategies such as selective overexpression of D2 in the human heart using a methodological approach as described earlier (125) could also be a strategy to restore local T3 levels. However, till now only D2 mRNA has been shown in the human heart (60) and although D2 activity was reported earlier in the murine heart (98) this approach yielded only a minimal increase in T3 levels in the murine heart. Therefore, this approach seems to be only of theoretical importance.

Since our studies were performed on total heart homogenates it is of obvious interest for future studies to identify the source of D3 activity in the failing heart. Besides cardiomyocytes, cardiovascular cells or cardiofibroblasts could be the source. Whereas D3 activity has been observed in skeletal muscle in critically ill adult patients and very premature infants (126, 127) and D3 activity has been identified in human infantile hemangiomas and in a vascular tumor yielding a clinical picture referred as consumptive hypothyroidism (128, 129), no D3 activity or mRNA has been identified in fibroblasts so far. In a recent paper, specific detection of D3 protein by immunocytochemical staining in Purkinje cells of chicken cerebellum was reported (130). In this study polyclonal antibodies were used. Western blot analysis detected a protein fragment corresponding to the expected molecular mass, whereas RT-PCR as well as Northern blot analysis confirmed the presence of D3 mRNA. This was the first study to identify D3 protein. This would be a reasonable approach for the heart as well. Such antibodies should be able to discern between the different deiodinases.

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CHAPTER 8

SUMMARY & SAMENVATTING

Summary

Thyroid hormone is important for energy metabolism, the metabolism of nutrients, inorganic ion fluxes and thermogenesis. Thyroid hormone is also essential for stimulation of growth and development of various tissues at critical periods including the central nervous system. Whereas in the adult thyroid hormone deficiency or excess may lead to an extensive array of clinical manifestations which are usually reversible with proper treatment, prolonged deficiency of thyroid hormones during development usually leads to irreversible damage, depending on specific timing of onset and duration of thyroid hormone deficiency. Therefore, thyroid hormone levels are strictly regulated and thyroid hormone metabolism is a key process in the regulation of thyroid hormone homeostasis. Deiodination is the most important metabolic pathway. Thyroid hormone is produced in the thyroid mainly as the biologically inactive precursor T4. In humans, only about 20% of the receptor active T3 is produced by the thyroid; most circulating T3 is derived from ORD of T4 by D1 activity in the peripheral tissues, mainly in the liver. Local T3, however, is derived from ORD of T4 by D2 activity. Therefore, ORD is regarded as an activating step. Since IRD of T4 and T3 results in the biological inactive compounds rT3 and T2, respectively, IRD is regarded as an inactivating step. Other metabolic pathways for iodothyronines are glucuronidation or sulfation of the phenol hydroxyl group and, to a minor extent, ether bond cleavage and oxidative deamination of the alanine side chain.

This thesis is divided in two parts. In the first part of the thesis structure-activity studies were performed to identify important amino acid residues in deiodinases. D1 was extensively studied in cat, pig and human, whereas D2 and D3 were studied in pig. Chapter 2 describes studies performed on cat D1. Cat D1 enzyme kinetics differed from human and rat D1 with regard to substrate preference, but was homologous to dog D1. By elucidating the molecular basis for the substrate selectivity of cat D1 we identified the residues in a region between amino acid 40 and 70 that are important for substrate interaction. The importance of these residues was confirmed by site-directed mutagenesis.

In Chapter 3 porcine D1, D2 and D3 were cloned. Our results showed that porcine and human D1-3 share the highest homology at the amino acid level. Furthermore, the catalytic

properties of the porcine deiodinases were virtually identical to those reported for human deiodinases.

In Chapter 4 we reported on alternative splicing, a formerly unknown feature for human D1, although alternative splicing has been described for D2. D1mRNA splice variants were identified in typical D1-containing tissues, i.e. liver, kidney and thyroid. All variants coded for shorter proteins and only two variants included the catalytic Sec residue. Whereas besides wild-type D1 two other variants have the C-terminal epitope used for generation of hD1-specific antibodies, immunoblots of human liver, thyroid and kidney showed only wild-type D1 protein, suggesting insignificant expression of the splice-variants at the protein level. The function of hD1 splice variation therefore remains to be established.

The second part of the thesis describes studies regarding the pathophysiological importance of deiodinases and their regulation. In Chapter 3 we studied regulation of D1-3 in porcine tissues after methimazole treatment and in thyrotoxicosis. Most remarkable were the induction of D2 activity in skeletal muscle and thyroid in methimazole treated pigs. The expression of D2 activity in these tissues is of interest for studies regarding the importance of D2 in (hypothyroid) humans.

The aim of the study described in Chapter 5 was to investigate the role of local thyroid hormone metabolism in the development of compensated hypertrophy and heart failure. Regarding the regulation of the key cardiac genes, heart failure and local hypothyroidism share a common denominator. Our hypothesis was, that besides important defects in the signal transduction system, i.e. down-regulation of TR or inhibition of T3 transport, local inactivation of T3 by D3 is of importance in the pathogenesis of pathological hypertrophy. We showed significant induction of D3 activity in RV heart failure. In this respect it is interesting that D3 is considered to be an oncofetal gene, because re-expression of various fetal genes is characteristic for cardiac hypertrophy. Therefore, we consider enhanced D3-expression to be part of such a fetal gene program that needs to be activated to allow cell growth.

To assess the precise role of induction of D3 activity in the pathogenesis of heart failure a time series analysis was performed during the development of either pathological hypertrophy or heart failure. These studies are described in Chapter 6. RV specific induction of D3 was only detected in heart failure and coincided with the start of the development of heart failure. Furthermore, a RV specific decrease in the ratio of tissue T3/T4 was observed in failing hearts. This decrease was RV specific and together with the observation of a reduction in RV T3 tissue levels in hypertrophic hearts despite normal serum T3 levels the conclusion

must be that this decrease cannot be attributed to low serum T3 levels, but is due to induction of local D3 activity. Our conclusions based on these studies were: 1) Induction of D3 contributes to a more pronounced local hypothyroid state in the failing heart, thereby contributing to the development of heart failure. 2) Altered TH metabolism contributes to SERCA2a gene suppression and since detoriation of Ca2+ homeostasis is thought to be critical in the transition from compensatory hypertrophy to heart failure we propose that prevention of the events resulting in a decrease in local TH levels could be beneficial to prevent myocardial impairment *in vivo*. 3) Shifts in gene expression of key cardiac proteins can occur in TH-independent fashion.

Samenvatting

Schildklierhormoon speelt een belangrijke rol in de regulatie van het basaal metabolisme, het metabolisme van nutriënten, anorganische ion fluxen en thermogenese. Schildklierhormoon is verder essentieel voor stimulatie van de groei en ontwikkeling van verschillende weefsels gedurende kritische periodes, waaronder het centraal zenuwstelsel. Waar een tekort of overmaat aan schildklierhormoon op volwassen leeftijd kan leiden tot tal van klachten, die gewoonlijk na een accurate behandeling herstellen, leidt een voortdurend tekort aan schildklierhormoon gedurende de ontwikkeling gewoonlijk tot onherstelbare schade. Dit hangt samen met het moment van optreden en met de duur van het tekort aan schildklierhormoon. Schildklierhormoon niveaus worden daarom binnen strikte grenzen gehouden en schildklierhormoon metabolisme speelt een sleutelrol in de regulatie van schildklierhormoon homeostase. Het belangrijkste metabole proces is dejodering. Schildklierhormoon wordt door de schildklier geproduceerd voornamelijk als de biologisch inactieve voorloper T4. In de mens wordt ongeveer 20% van het receptoractieve T3 geproduceerd door de schildklier. Serum T3 wordt uit T4 gevormd, voornamelijk door in de lever gelokaliseerde D1 activiteit. Lokaal T3 daarentegen wordt gevormd door D2 activiteit. Omdat dit in beide gevallen buitenring dejodering betreft, beschouwt men deze vorm van dejodering ook wel als activerend. Binnenring dejodering van T4 en T3 daarentegen leidt tot productie van de biologisch inactieve substraten rT3 en T2. Daarom beschouwt men deze vorm van dejodering ook wel als inactiverend. Naast dejodering zijn er nog andere processen betrokken bij het metabolisme van jodothyronines, zoals glucuronidering of sulfatering van de phenolische hydroxyl groep en, in mindere mate, scheiding van de ether verbinding en oxidatieve deaminering van de alanine zijketen

Dit proefschrift is in twee delen verdeeld. In het eerste deel worden structuur-activiteit studies beschreven die gedaan zijn om belangrijke aminozuren in dejodases te identificeren. We hebben D1 uitgebreid bestudeerd in de kat, het varken en de mens en D2 en D3 in het varken. Hoofdstuk 2 beschrijft studies met betrekking tot katten-D1. De kinetiek van dit enzym verschilde van humaan and ratten-D1 wat betreft substraat preferentie, maar was daarentegen gelijk aan die van honden-D1. Door de moleculaire basis van de substraat-selectiviteit van katten-D1 op te helderen, identificeerden we aminozuur residuen gelegen in een regio tussen aminozuur 40-70, die van belang zijn voor substraat interactie. Mutatie studies bevestigden het belang van deze residuen.

In hoofdstuk 3 werden D1, D2 and D3 in het varken gekloneerd. Onze resultaten lieten zien dat D1-3 in het varken de hoogste homologie met humaan D1-3 vertonen op aminozuur niveau in vergelijking met andere species. Ook de enzymkarakteristieken van de varkens dejodases waren nagenoeg gelijk aan die van de humane dejodases.

In hoofdstuk 4 beschreven we het voorkomen van D1 mRNA varianten in humane weefsels. Deze mRNA varianten werden eerder beschreven voor D2. D1mRNA varianten kwamen voor in typische D1 weefsels zoals lever, nier en schildklier. Elke variant codeerde voor een korter D1 eiwit, maar slechts 2 varianten bezaten het katalytische Sec residu. Hoewel naast wild-type D1 2 varianten de C-terminale epitoop bevatten, die we hebben gebruikt voor de productie van hD1-specifieke antilichamen, laten immunoblots van de humane lever, schildklier en nier alleen maar wild-type D1 eiwit zien. Dit suggereert dat deze 2 varianten niet significant tot expressie komen op eiwit niveau. De functie van dergelijke hD1 mRNA varianten moet derhalve worden onderzocht.

In het tweede deel van dit proefschrift hebben we gekeken naar de rol van dejodases in pathofysiologische omstandigheden alsmede de regulatie van D1-D3. In hoofdstuk 3 bestudeerden we de regulatie van D1-3 in weefsels van het varken na behandeling met methimazol en gedurende thyrotoxicose. Het meest opvallende was de inductie van D2-activiteit in skeletspier en schildklier in methimazol behandelde varkens. Het feit, dat er expressie van D2 activiteit in deze weefsels is, is belangrijk voor studies die de importantie van D2 in (hypothyreote) mensen onderzoeken.

Het doel van de studie beschreven in hoofdstuk 5 was om de rol van lokaal schildklierhormoon metabolisme te onderzoeken bij de ontwikkeling van gecompenseerde hypertrofie en hartfalen. Met betrekking tot de regulatie van belangrijke cardiale genen laten hartfalen en een locale hypothyreoïdie een zelfde patroon zien. Onze hypothese was, dat naast belangrijke defecten in de T3-signaal transductie, dat wil zeggen down-regulering van de TR

of remming van het T3 transport over de celmembraan, locale inactivatie van T3 door D3 van belang is in the pathogenese van pathologische hypertrofie. We lieten significante inductie van D3 activiteit in het RV van het falende hart zien. In dit verband is het vermeldenswaardig, dat D3 wordt beschouwd als een oncofoetaal gen. Re-expressie van tal van foetale genen is een aspect dat karakteristiek is voor cardiale hypertrofie. We beschouwen de verhoogde D3-expressie als onderdeel van zo'n foetaal gen programma, dat geactiveerd dient te worden, zodat celgroei kan plaatsvinden.

Om de exacte rol van inductie van D3 activiteit in de pathogenese van hartfalen vast te stellen werden er analyses gedaan op verschillende tijdstippen in de ontwikkeling van pathologische hypertrofie en hartfalen. Deze studies staan beschreven in hoofdstuk 6. RV specifieke inductie van D3 werd alleen gemeten in hartfalen en valt samen met de start van het ontwikkelen van hartfalen. Verder was er alleen in de falende harten sprake van een RV specifieke daling in de weefsel T3/T4 verhouding. Samen met de observatie van een gedaald RV weefsel T3 in hypertrofische harten ondanks normale serum T3 waarden betekent dit, dat deze daling niet kan worden toegeschreven aan lage serum T3 waarden, maar dat dit veroorzaakt wordt door inductie van locale D3 activiteit. Onze conclusies gebaseerd op deze studies waren: 1) Inductie van D3 draagt bij aan een meer uitgesproken locale hypothyreoïdie in het falende hart en dit draagt bij aan de ontwikkeling tot hartfalen. 2) Veranderd schildklierhormoon metabolisme draagt bij aan SERCA2a gene suppressie. Gezien het feit, dat verslechtering van Ca2+ homeostase wordt gezien als een kritische factor in de overgang van compensatoire hypertrofie naar hartfalen stellen we, dat wanneer gebeurtenissen resulterend in een daling van lokaal T3 kunnen worden voorkomen dit nuttig is om myocardiale verslechtering *in vivo* te voorkomen. 3) Verschuivingen in genexpressie van belangrijke cardiale eiwitten kunnen ook onafhankelijk van T3 plaatsvinden.

CURRICULUM VITAE

Franciscus Wilhelmus Johannes Stephan Wassen werd op 10 juli 1971 geboren te Venlo. Het Gymnasium-ß diploma behaalde hij in 1989 aan het Sint Thomascollege te Venlo. In september van dat jaar begon hij aan de studie geneeskunde aan de Erasmus Universiteit te Rotterdam waar hij in 1995 het doctoraalexamen behaalde. Het afstudeeronderzoek verrichtte hij van 22 augustus 1994 tot 1 augustus 1995 op de afdeling Inwendige Geneeskunde van de Erasmus Universiteit onder leiding van mw. dr. M.E. Everts. Het onderwerp betrof het transport van schildklierhormoon in de hypofyse, te weten de rol van ATP en effecten van hormonen of verbindingen die in het serum van patiënten voorkomen tijdens non-thyroidal illness (NTI). Dit leverde naast twee publicaties in vooraanstaande wetenschappelijke tijdschriften een Eervolle Vermelding op ter gelegenheid van het 25-jarig bestaan van de Gerrit Jan Mulder Stichting. In januari 1998 behaalde Frank met lof het artsexamen. In november 1998 startte hij op de afdeling Inwendige Geneeskunde van het Erasmus MC als Assistent Geneeskundige In Opleiding tot Klinisch Onderzoeker (AGIKO), een traject waarin de opleiding tot internist wordt volbracht alsmede promotieonderzoek wordt verricht. De klinische opleiding stond aanvankelijk onder leiding van Prof.dr. S.W.J. Lamberts en later onder die van Prof.dr. H.A.P. Pols. Het in dit proefschrift beschreven promotieonderzoek werd verricht op de afdeling Inwendige Geneeskunde van de Erasmus MC onder leiding van Prof.dr.ir. T.J. Visser. Aan de totstandkoming van dit proefschrift hebben meegewerkt de afdeling Experimentele Cardiologie van de Erasmus Universiteit onder leiding van Prof.dr. D.J.G.M. Duncker alsmede het Instituut voor Cardiovasculaire Research van de faculteit fysiologie van de Vrije Universiteit van Amsterdam (dr. W.S. Simonides en dr. A.E. Schiel). Inmiddels heeft Frank's carrière een wending genomen en is hij sinds 17 mei 2004 werkzaam als keurend arts bij een Arbo-dienst. Vanaf september 2005 zal hij de opleiding tot Bedrijfsarts gaan volgen.

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Stellingen

Behorende bij het proefschrift

IODOTHYRONINE DEIODINASES: STRUCTURE-FUNCTION ANALYSIS AND THEIR ROLE IN THE REGULATION OF THYROID HORMONE LEVELS

1. Een falend hart is een hart met een lokaal tekort aan schildklierhormoon. *Dit proefschrift*

2. Vergelijkende sequentie analyse en mutatie analyse vormen een bruikbaar alternatief voor structuur-functie onderzoek van membraan gebonden en derhalve niet oplosbare eiwitten, waar de dejodasen een voorbeeld van zijn. *Dit proefschrift*

3. D1 activiteit is belangrijk voor serum T3 productie en D2 activiteit voor de productie van lokaal T3. De bevinding van een hoge D2 activiteit in skeletspieren onder hypothyreote omstandigheden suggereert dat onder deze condities D2 activiteit in de skeletspier tevens kan bijdragen aan serum T3 productie. *Dit proefschrift*

4. Het D3 gen is een oncofoetaal gen en is een van de genen behorende tot het foetale genpatroon, dat bij hartfalen tot reëxpressie wordt gebracht. *Dit proefschrift*

5. Hartfalen gaat gepaard met lokale hypothyreoïdie. Suppletie van actief schildklier-hormoon (T3) lijkt echter geen optie te zijn, daar het lokaal geactiveerde D3 het T3 in het hart direct zal afbreken. Het lijkt dan ook zinvoller T3 analoga te ontwikkelen, die resistent zijn tegen de inactiverende werking van D3 of weefselspecifieke D3 remmers te ontwerpen. *Dit proefschrift*

6. "Donner aux uns, cela veut toujours dire prendre aux autres". Uit *Les Pensees*-Wolinski.

7. De uitspraken "ieder nadeel heeft zijn voordeel" en "toeval is logisch" (uit *Je moet schieten anders kun je niet scoren en andere citaten van JC Cruijff* - Henk Davidse) kenschetsen het vak van onderzoeker.

8. "Alles van waarde is weerloos"-Lucebert

9. Europa bestaat uit een diversiteit aan volkeren. De uitspraak "Europa is een schilderij maken en geen som oplossen" – uit *Referendum campagne Loesje 19 april 2005* geeft dit goed weer. Je stuit onherroepelijk op gevoeligheden wanneer je hiermee geen rekening houdt bij inspanningen om één economisch blok tegen Amerika en Azië te vormen.

10. De manier waarop onze samenleving de huidige problematiek in het sociale stelsel te lijf gaat, zoals het de gehele dag vastbinden van demente ouderen op hun bed en het laten doorwerken van oudere, pensioengerechtigde werknemers tot ze er dood bij neervallen, is een West-Europese samenleving als de onze onwaardig.

11. " Health checks " zijn zeer populair in onze huidige samenleving, omdat ze inspelen op de behoefte van ieder individu om zoveel mogelijk geinformeerd te raken over zijn huidige gezondheidstoestand. Men dient echter de mensen te informeren, dat aan ieder medische test die men verricht een fout-positief of een fout-negatief percentage kleeft. Door dit na te laten creëert men met iedere uitspraak over de gezondheidstoestand een schijnzekerheid en daar is niemand bij gebaat.