Chapter 4
Regulation of Blood Vessels by Prolactin and Vasoinhibins

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Abstract Prolactin (PRL) stimulates the growth of new blood vessels (angiogenesis) either directly through actions on endothelial cells or indirectly by upregulating pro-angiogenic factors like vascular endothelial growth factor (VEGF). Moreover, PRL acquires antiangiogenic properties after undergoing proteolytic cleavage to vasoinhibins, a family of PRL fragments (including 16 kDa PRL) with potent antiangiogenic, vasoconstrictive, and antivasopermeability effects. In view of the opposing actions of PRL and vasoinhibins, the regulation of the proteases responsible for specific PRL cleavage represents an efficient mechanism for controlling blood vessel growth and function. This review briefly describes the vascular actions of PRL and vasoinhibins, and addresses how their interplay could help drive biological effects of PRL in the context of health and disease.

4.1 Introduction

Prolactin (PRL) is remarkably versatile, as it regulates various events in reproduction, osmoregulation, growth, energy metabolism, immune response, brain function, and behavior [1–3]. Blood vessels are emerging as PRL targets contributing to these actions [4]. By transporting fluid, nutrients, oxygen, hormones, growth factors, cytokines, immune cells, and waste material, the vascular system helps regulate most if not all body functions including growth, energy homeostasis, inflammation, and brain activity. PRL stimulates or inhibits the proliferation, dilation, permeability, and regression of blood vessels. These opposing effects reside within the PRL molecule as the full-length protein promotes angiogenesis, but after proteolytic processing the resulting PRL fragments, vasoinhibins, exert antiangiogenic, vasoconstrictive, and antivasopermeability effects (Fig. 4.1). The combination of these stimulatory and inhibitory properties can lead to differences in the perfusion of target tissues, thereby influencing their growth and function. In this review, we

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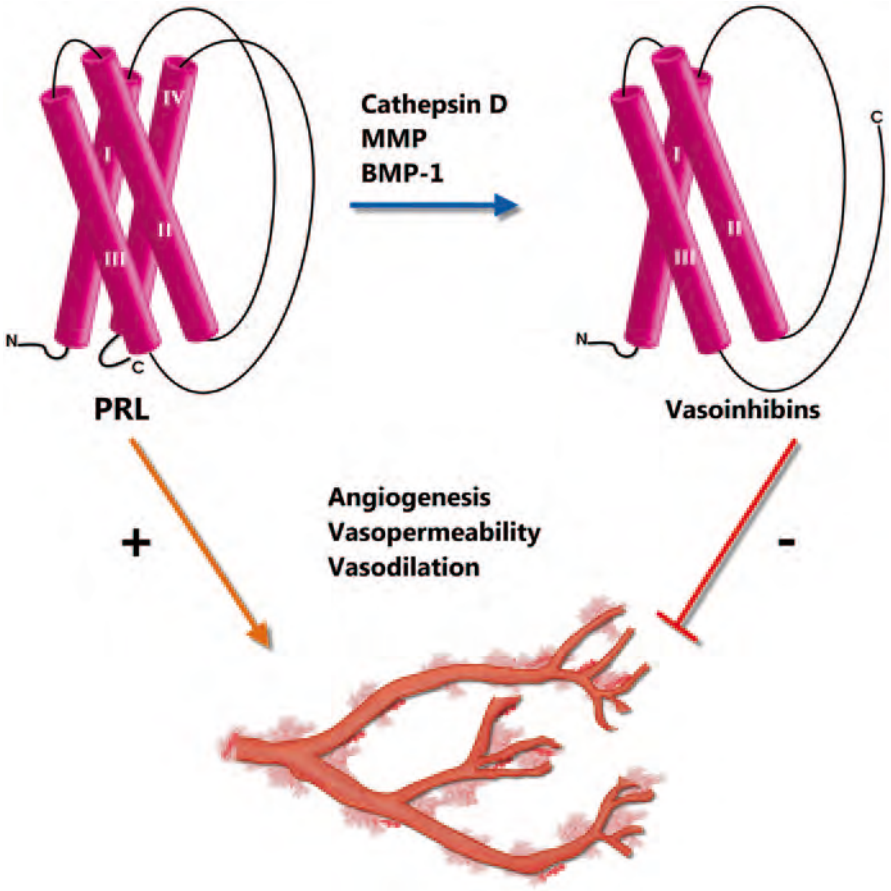

Fig. 4.1 *PRL* stimulates blood vessel growth and function and acquires vascular inhibitory properties after undergoing specific proteolytic cleavage to vasoinhibins by cathepsin D, matrix metalloproteases (MMP), and bone morphogenetic protein-1 (BMP-1)

clarify the advantage of the vasoinhibin nomenclature, and concisely address the generation of vasoinhibins, the effects of PRL and vasoinhibins on blood vessels, and how these vascular actions could affect tissue growth, function, and involution under normal and pathological conditions.

4.2 The Vasoinhibin Term

PRL fragments with inhibitory effects on blood vessels were originally termed "16 kDa PRL." The initial paper by Ferrara et al. [5] used a purified fraction of enzymatically cleaved rat PRL (having 145 amino acids and 16.3 kDa) that showed

an inhibitory effect on endothelial cell proliferation. However, follow-up studies, confirming and extending these vascular actions, used recombinant PRL fragments of different sizes that were still named 16 kDa PRL in an attempt to maintain the connection to the original preparation. Indeed, various laboratories used a recombinant fragment of 14 kDa containing the first 123 amino acids of human PRL [6–8], the same fragment but coupled to a polyhistidine tail [9, 10], a 15.6 kDa fragment containing the first 139 amino acids of human PRL [11–13], or a 17.2 kDa fragment containing the first 150 amino acids of human PRL [14]. The term 16 kDa PRL became even less accurate when studying the endogenous peptides generated by specific proteases. It then became evident that PRL fragments with inhibitory effects on blood vessels are not a single 16 kDa species, but rather a family of peptides with different molecular masses so far ranging from 14 to 18 kDa and all sharing the N-terminal region of PRL.

Different proteases generate the various fragments by cleaving at different sites near or within the long loop connecting the third and fourth α-helices of the PRL molecule (Fig. 4.1). Cathepsin D cleaves rat PRL and mouse PRL into a 16 kDa N-terminal fragment [15, 16], bovine PRL into 14 and 16 kDa N-terminal fragments [17], buffalo PRL into 11, 14, and 18 kDa antiangiogenic fragments [18], and human PRL into 11, 15, 16.5, and 17 kDa N-terminal fragments [19]. Matrix metalloproteases (MMP) predominantly cleave human and rat PRL at amino acids 155 and 153, respectively, to generate 17 kDa N-terminal fragments [20], and bone morphogenetic protein-1 (BMP-1) cleaves after the first 159 amino acids of human and mouse PRL, generating an 18 kDa fragment [21]. Since these peptides share blood vessel inhibitory properties, in 2006 we proposed the term “vasoinhibins” [20, 22] to refer to the whole family of PRL-derived N-terminal fragments that have inhibitory vascular effects. All PRL N-terminal fragments of 14 to 18 kDa that have been tested to date demonstrate blood vessel inhibitory properties, reinforcing the concept that this structure is responsible for the vasoinhibin identity.

4.3 Generation of Endogenous Vasoinhibins

The fact that vasoinhibins are produced by different proteases implies that their generation can occur under different conditions and tissue microenvironments. Cathepsin D is catalytically active at acidic pH (pH < 5.5), and recent findings showed that it is the main vasoinhibin-generating enzyme in anterior pituitary lactotrophs [23]. Cathepsin D is located in PRL secretory granules, which are acidic, and cathepsin-D null mice are devoid of vasoinhibins in the anterior pituitary gland [23]. Accordingly, vasoinhibins can be generated by cathepsin D during the pituitary PRL secretory process and thus, subjected to regulated release. Along this line, estradiol increases the synthesis and activity of cathepsin D [24], and the production of anterior pituitary vasoinhibins is higher in females [23], increases at proestrus with respect to diestrus, and estrogens stimulate their release [25].

Cathepsin D may also be released from secretory granules or lysosomes at anterior pituitary or extrapituitary locations to generate vasoinhibins outside cells. Cathepsin D cleaves PRL in the extracellular milieu of regressing corpus luteum [17] and mammary gland [16], and in cardiomyocytes under oxidative stress [13], conditions in which tissue remodelling and altered metabolic activity can acidify the pericellular pH. Cultured GH4C1 pituitary adenoma cells also secrete cathepsin D, and mimicking the tumor microenvironment by exposure to hypoxia reduces its release [26], suggesting that extracellular production of vasoinhibins could be decreased and favor the proangiogenic condition necessary for prolactinoma growth.

On the other hand, PRL may be physiologically cleaved outside cells by the extracellular matrix-degrading enzymes, MMP and BMP-1, which act at neutral pH and are secreted or anchored to the external cell surface. MMP and BMP-1 released by chondrocytes [20] and embryonic fibroblasts [21], respectively, generate vasoinhibins from PRL, a mechanism that may serve to maintain cartilage avascularity and to limit developmental angiogenesis. MMP and BMP-1 also produce other antiangiogenic factors by proteolytic processing [27, 28]; however, both types of proteases are upregulated in diseased states characterized by blood vessel growth and invasion [29, 30]. As high concentrations of MMP also lead to the degradation of both PRL and vasoinhibin [20], in some cases MMP upregulation may downregulate vasoinhibins to favor pathological angiogenesis.

Consistent with the ubiquitous nature of PRL-cleaving enzymes, endogenous vasoinhibins have been identified in the anterior [23] and posterior pituitary gland [31], hypothalamus [32], cartilage [20], retina [33], cardiomyocytes [13], corpus luteum [17], mammary gland [16], and in biological fluids (serum, amniotic fluid, and urine) [34, 35].

4.4 Vascular Effects of PRL and Vasoinhibins

The effects and signaling mechanisms of PRL and vasoinhibins on blood vessels have been extensively reviewed [4, 22, 36]. Here, we will briefly summarize previous findings with a focus on recent advances. PRL stimulates angiogenesis during development (chick chorioallantoic membrane, CAM) and in adult tissues (corpus luteum, testis, and heart). These observations were recently extended to include the angiogenesis of transplanted pancreatic islets [37] and the neovascularization associated with normal and regenerative liver growth, where inducing hyperprolactinemia increased hepatic endothelial cell proliferation and vascular endothelial growth factor (VEGF) expression [38, 39]. Moreover, in addition to the known effects of PRL on endothelial cell proliferation and VEGF expression, PRL was recently shown to stimulate the migration and tube formation of endothelial cells [40, 41], to reduce vasopermeability by upregulating the expression of tight-junction proteins between endothelial cells [42], and to promote intussusceptive angiogenesis in the CAM [40]. The latter differs from sprouting angiogenesis in that new blood vessels are formed by the splitting of an existing blood vessel in two, which is essentially independent of endothelial cell proliferation and thereby, less energy demanding [43].

PRL can promote angiogenesis by direct actions on endothelial cells. However, the effects of PRL on cultured endothelial cells are modest and not always observed [40, 41, 36]. PRL actions may be limited by underexpressed PRL receptors in endothelial cells. Exposure to ovarian follicular fluid stimulates the expression of the long and short PRL receptor isoforms in bovine umbilical vein endothelial cells (BUVEC), and PRL does not stimulate BUVEC proliferation unless the cells are pretreated with ovarian follicular fluid [44]. In addition, vascular endothelial cells produce and release PRL, so the locally produced hormone may limit the effects of exogenous PRL by occupying its receptors in endothelial cells. Yang and colleagues recently highlighted the role of PRL as an autocrine regulator of angiogenesis. They showed that PRL produced by endothelial cells is a downstream mediator of STAT5-induced endothelial cell migration, invasion, tube formation, and VEGF expression [41]. The fact that STAT5 mediates these angiogenesis events in response to fibroblast growth factor-2 (FGF-2) [45] places PRL in the signaling cascade of potent angiogenesis stimulators. Also, PRL itself activates STAT5 in endothelial cells [40, 41] and stimulates the expression of FGF-2 and VEGF by various nonendothelial cell types [4], suggesting that PRL acts as a positive autocrine and paracrine feedback regulator of angiogenesis.

The complexity of the vascular effects of PRL is further illustrated by conflicting data showing that PRL is unable to stimulate angiogenesis in the mouse cornea, that siRNA-targeting PRL results in angiogenesis in the rat retina, and that disruption of the PRL gene is associated with highly vascularized pituitary tumors in aged mice [4, 36]. Moreover, PRL has opposing effects on vascular resistance, blood volume, and blood flow that depend on the experimental model and conditions [36]. These inconsistencies may involve the proteolytic conversion of PRL to vasoinhibins.

Vasoinhibins inhibit angiogenesis, vasodilation, and vasopermeability, and promote vascular regression in the cornea, retina, heart, and xenografted tumors. They act directly on endothelial cells to inhibit the action of several vasoactive substances including: VEGF, FGF-2, interleukin 1-β, bradykinin, and acetylcholine. Vasoinhibins signal through a still-unidentified receptor distinct from the PRL receptor: (1) to cause cell cycle arrest by blocking activation of the MAPK pathway at the level of Ras, decreasing cyclin D1, and upregulating p21; (2) to inhibit endothelial cell migration by increasing type-1 plasminogen activator inhibitor and thus reducing urokinase activity, and by downregulating the Ras-Tiam1-Rac1-Pak1 pathway; and (3) to induce endothelial cell apoptosis by promoting NFκB-mediated caspase-8 and 9 activation, which in turn stimulate caspase-3 and DNA fragmentation. In addition, vasoinhibins were recently shown to induce the expression of microRNA-146a (miR-146a) in endothelial cells in an NFκB-dependent manner [46]. Silencing miR-146a expression prevented the inhibitory effects of vasoinhibins on endothelial cell proliferation and survival, but not on endothelial cell migration, revealing miR-146a as a mediator of a large fraction of vasoinhibins antiangiogenic effects.

Another key mechanism by which vasoinhibins regulate endothelial cell function, specifically causing vasoconstriction and reduced vasopermeability, is by blocking the activation of endothelial nitric oxide synthase (eNOS). They do so by promoting protein phosphatase 2 A-induced dephosphorylation and inactivation of eNOS, by blocking the activation of phospholipase C and the formation of inositol

1,4,5-triphosphate leading to a reduced release of Ca^{2+} from intracellular stores, and by interfering the expression of transient receptor potential canonical (TRPC) channels [47, 4]. Also, dephosphorylation-mediated inactivation of eNOS can contribute to vasoinhibin inhibition of endothelial cell proliferation and migration. Vasoinhibins block the increase in eNOS activity, migration, and proliferation of endothelial cells overexpressing wild type eNOS but did not affect these responses in cells overexpressing phosphomimetic or nonphosphorylatable eNOS mutants [48].

Besides inhibiting eNOS-mediated vasodilation, vasoinhibins can lower blood flow in developing blood vessels by reducing pericyte coverage of capillaries [49]. Vasoinhibins interfere with pericyte recruitment by disrupting the Notch signaling pathway in endothelial cells, and this action can lead to a dysfunctional vasculature in a murine melanoma tumor model [49]. Finally, vasoinhibins exert proinflammatory actions on blood vessels; they stimulate leukocyte adhesion to endothelial cells and leukocyte infiltration into tumors by activating NFκB and increasing the expression of adhesion molecules in endothelial cells [50]. These actions may also involve vasoinhibin-induced downregulation of eNOS, since VEGF stimulation of eNOS-mediated NO production promotes endothelial cell anergy [51].

In spite of the abundance of data concerning the vascular actions and signaling mechanisms of vasoinhibins, the nature of the vasoinhibin receptor remains unresolved. More than two decades ago, vasoinhibins were shown to bind to a single class of sites on endothelial cell membranes (Kd of 1–10 nM), associating with proteins of 52 and 32 kDa that were distinct from the PRL receptor [52]. Whether these represented receptors or regulatory binding proteins important for vasoinhibin functions is unknown. Difficulties in identifying the vasoinhibin receptor(s) may lie in that they could be forming a complex with other receptors and binding proteins. Similar receptor complexes have been proposed for angiostatin and endostatin, that are also families of antiangiogenic peptides derived by proteolysis from precursor proteins [53, 54].

4.5 Contribution of Blood Vessel Regulation to PRL Biological Effects

The influence of the vascular actions of PRL and vasoinhibins on the regulation of PRL target organs (crop-sac, mammary gland, corpus luteum, retina, cartilage, and heart) has been previously reviewed in a physiopathological [4, 36, 55, 56] and evolutionary [57] context. Here, we extend this discussion by addressing recent findings and promising new avenues.

4.5.1 Mammary Gland

The mammary gland stands as a major PRL target organ. PRL stimulates the growth, differentiation, milk production, and survival of mammary epithelium.

These events are dependent on the expansion and regression of the mammary gland vasculature [58], which may be influenced by PRL and vasoinhibins. PRL promotes the expression of VEGF in mammary epithelial cells [36], and weaning upregulates the expression and activity of the vasoinhibin-generating proteases, MMP, and cathepsin D [59]. Vasoinhibins were recently detected in mouse mammary glands, and their levels increased during involution together with those of the mature cathepsin D isoform [16]. Moreover, PRL stimulates the activation and polarized secretion of cathepsin D by mammary tissue [60]. This mechanism may help attenuate vascular expansion during lactation and promote blood vessel regression during involution since PRL expression and cathepsin D-mediated PRL cleavage increase in the lactating and regressing mammary gland [16, 36].

However, the mechanisms regulating the antiangiogenic effects of vasoinhibins may be altered in the malignant state. Neoplastic breast tissue shows diminished vasoinhibin-generating activity [61] and higher levels of PRL receptors in cells [62], including those of the microvasculature [40]. In contrast to the reduced growth observed in prostate, colon, and melanoma tumors expressing vasoinhibins, tumors derived from breast cancer cells induced to produce vasoinhibins exhibit decreased vascularization but no effect on tumor size [14]. This is surprising as vasoinhibins have the ability to inhibit and promote the growth [14] and apoptosis [16] of breast cancer cells, respectively.

4.5.2 Corpus Luteum

Similar to the mammary gland, the corpus luteum undergoes dramatic expansion and involution at the expense of the vasculature. In rodents, PRL is luteotrophic in pregnancy but luteolytic during nonfertile cycles. These opposing effects may reflect in part the vascular interplay between PRL and vasoinhibins. PRL stimulates the proliferation of endothelial cells in the corpus luteum, and lowering systemic PRL or disrupting the PRL receptor interferes with corpus luteum neovascularization. By using transgenic mice expressing only the long form of the PRL receptor, PRL-induced stimulation of VEGF production and neovascularization of the corpus luteum was specifically linked to the short form of the PRL receptor [63], which is the predominant form found in corpus luteum endothelial cells [64].

4.5.3 Retina

In contrast to reproductive organs, the vasculature is dormant throughout life in most adult tissues and is highly restricted in cases such as the retina. Vasoinhibins help maintain the quiescent state of retinal blood vessels and protect against aberrant vasopermeability and angiogenesis in retinopathy of prematurity and diabetic retinopathy. Retinal vasoinhibins may derive from PRL synthesized in the retina and from systemic PRL accessing the eye via its receptors in the ciliary body [65].

Hyperprolactinemia increases the levels of retinal vasoinhibins, which in turn reduce VEGF and diabetes-induced retinal vasopermeability [65]. Similarly, the transfer to the retina of the vasoinhibin gene via adenoassociated virus type 2 vectors prevents vascular alterations associated with nonproliferative diabetic retinopathy [66].

4.5.4 Heart

Accumulating evidence has linked vasoinhibin overproduction to the pathophysiology of peripartum cardiomyopathy. Increased oxidative stress causes cathepsin D-mediated PRL cleavage to vasoinhibins, which in turn interfere with the growth and function of coronary vasculature required for adequate performance of the maternal heart during pregnancy and lactation. MiR-146a, discovered as a major mediator of vasoinhibin antiangiogenic actions, is also responsible for vasoinhibin effects causing myocardial metabolic dysfunction [46]. Vasoinhibins stimulate the shedding from endothelial cells of exosomes loaded with mirR-146a that, when absorbed by cardiomyocytes, impairs their metabolic activity [46]. Altogether, these concepts have led to the development of promising combination therapies employing bromocriptine and to the evaluation of markers (cathepsin D activity and miR-146a serum levels) for diagnosis and disease monitoring [67].

4.5.5 Other

Other promising research directions relate to the liver, pancreas, and brain. Liver growth is angiogenesis-dependent and coincides with the hyperprolactinemia ocurring during pregnancy and lactation, cirrhosis [68], and after partial hepatectomy [69]. Absence of the PRL receptor confers reduced liver mass, and elevating systemic PRL promotes growth and neovascularization of the normal and regenerating adult liver [38]. During pregnancy, the need for insulin action results in pancreatic islet growth, which is angiogenesis dependent. PRL and placental lactogens stimulate the proliferation, survival, and insulin production by pancreatic β-cells [70], and PRL stimulates vascular density and downregulates the expression of the angiogenesis inhibitor thrombospondin-1 (TSP-1) in transplanted pancreatic islets [37]. Moreover, chronic exposure of isolated human islets to high glucose concentrations impairs angiogenesis, reduces PRL and MMP-9 expression, and increases TSP-1 synthesis [71]. These findings suggest that PRL mediates pancreatic islet neovascularization and growth during pregnancy, and that an altered production of PRL and vasoinhibins may impact abnormal islet angiogenesis in diabetes. PRL acts in the brain to stimulate neurogenesis and neuronal survival [3], which are effects frequently elicited by proangiogenic substances [72]. PRL also reduces the permeability of brain capillary endothelial cells in a NO-independent manner [42], and vasoinhibins inhibit NO-dependent vasopermeability in the retina, thus suggesting that the PRL-vasoinhibin system helps maintain the brain- and retinal-blood

barriers. Finally, exposure to stress reduces the conversion of PRL to vasoinhibins in the hypothalamus, and the intracerebroventricular administration of PRL and vasoinhibins attenuates and enhances stress-related behaviors (anxiety and depression) [32], respectively; these behaviors associate with altered cerebral blood flow and endothelial cell dysfunction [73].

Concluding Remarks

The vascular effects of PRL and vasoinhibins are emerging as novel mechanisms balancing growth, function, and involution. Further research is needed to clarify the regulation of the specific proteases, the receptors, and signaling pathways involved, and how PRL and vasoinhibins interact to affect blood vessel and organ function under health and disease.

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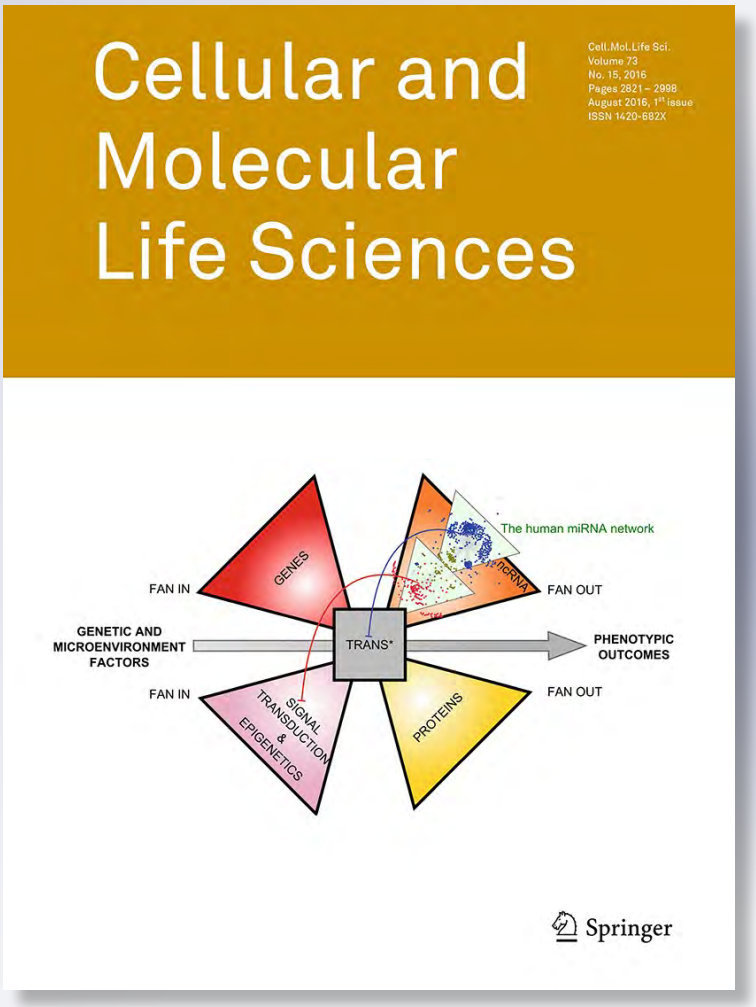

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Cellular and Molecular Life Sciences

REVIEW

The role of the prolactin/vasoinhibin axis in rheumatoid arthritis: an integrative overview

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Abstract Rheumatoid arthritis (RA) is a chronic, autoimmune, inflammatory disease destroying articular cartilage and bone. The female preponderance and the influence of reproductive states in RA have long linked this disease to sexually dimorphic, reproductive hormones such as prolactin (PRL). PRL has immune-enhancing properties and increases in the circulation of some patients with RA. However, PRL also suppresses the immune system, stimulates the formation and survival of joint tissues, acquires antiangiogenic properties upon its cleavage to vasoinhibins, and protects against joint destruction and inflammation in the adjuvant-induced model of RA. This review addresses risk factors for RA linked to PRL, the effects of PRL and vasoinhibins on joint tissues, blood vessels, and immune cells, and the clinical and experimental data associating PRL with RA. This information provides important insights into the pathophysiology of RA and highlights protective actions of the PRL/vasoinhibin axis that could lead to therapeutic benefits.

Keywords Gender · Stress · Reproduction · Angiogenesis · Joint tissues · Immune cells · Cartilage · Bone · Blood vessels

Abbreviations

RA	Rheumatoid arthritis
PRL	Prolactin
ACPA	Anti-citrullinated peptide antibodies
RF	Rheumatoid factor
TRAF1-C5	Tumor necrosis factor receptor-associated factor 1
STAT	Signal transducer and activator of transcription
NF-κβ	Nuclear factor κβ
Th1	T helper 1
Th2	T helper 2
IL	Interleukin
IFNγ	Interferon γ
TNF α	Tumor necrosis factor α
JAK	Janus kinase
PI3k	Phosphatidylinositol 3-kinase
MAPK	Mitogen-activated protein kinase
BMP-1	Bone morphogenetic protein-1
RANKL	Receptor activator of NF-κβ ligand
VEGF	Vascular endothelial growth factor
FGF-2	Fibroblast growth factor-2
HO-1	Heme oxigenase-1
BK	Bradykinin
ACh	Acetylcholine
PAI-1	Plasminogen activator inhibitor-1
uPA	Urokinase plasminogen activator
uPAR	Urokinase plasminogen activator receptor
eNOS	Endothelial nitric oxide synthase
PP2A	Protein phosphatase 2A

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TRPC5	Transient receptor potential channel 5
PRLR	Prolactin receptor
NK	Natural killer
iNOS	Inducible nitric oxide synthase
NO	Nitric oxide
TRH	Thyrotropin-releasing hormone
TIMP-1	Tissue inhibitor of MMP
CFA	Complete Freunds adjuvant
AP	Anterior pituitary gland

Introduction

Rheumatoid arthritis (RA) is a disabling disease affecting nearly 1 % of the adult population worldwide with a female:male ratio of 3:1. The causes of RA remain unknown but may involve a combination of genetic, environmental, and host factors that initiate the response to an unidentified trigger and result in the recruitment of immune cells into the joints. Production of autoantibodies, inflammatory interactions between activated immune cells and synoviocytes, and synovial angiogenesis sustain and amplify the inflammatory reaction. Chronic inflammation, invasion, cell death, and proteolytic matrix degradation destroy the adjacent cartilage and bone and lead to systemic complications and increased comorbidities.

Among host factors able to affect the pathophysiology of RA is the sexually dimorphic hormone, prolactin (PRL). PRL is a stress-related [1, 2] and reproductive hormone, essential for lactation, that regulates a wide diversity of processes [3–5] including events in joint tissues and immune cells. PRL regulates cartilage survival [6, 7], bone formation and resorption [6, 8], the growth of blood vessels [9], the proliferation, survival, and function of immune cells [10–12], and the progression of rheumatic diseases [6, 10, 11]. Some of these actions are affected by the proteolytic conversion of PRL to vasoinhibins, a family of PRL fragments with effects opposite to those of PRL on inflammation and angiogenesis [13]. The generation, secretion, and action of PRL and vasoinhibins are integrated at the hypothalamus, the pituitary, and the target tissue levels and this organization has been recently defined as the PRL/vasoinhibin axis [14]. In this review, we briefly summarize current knowledge of risk factors for RA that can be linked to PRL, the effects of PRL and vasoinhibins on joint tissues, blood vessels, and immune cells, and the experimental and clinical evidences associating PRL with RA. We propose that the ability of the PRL/vasoinhibin axis to exert opposing effects on inflammatory reactions and angiogenesis contributes to the controversial role of PRL on the pathophysiology of RA and offers opportunities for the development of new treatments.

RA essentials

RA is the most common form of inflammatory arthritis; it occurs at any age but its incidence is higher among those aged 40–70 years. Chronic synovitis underlies the primary manifestations of this disease, including pain, stiffness, and swelling, which are eventually followed by cartilage destruction, bone erosion, subsequent joint deformities, and systemic complications.

The detailed essentials of RA are beyond the scope of this article and can be found in several reviews [15–18]. A major challenge is that RA manifests in distinct disease subsets with different phenotype, fluctuating course, and uncertain progression. While some patients show a mild development that can be self-limiting, others acquire severe, erosive disease with systemic manifestations. Nonetheless, it is increasingly clear that the outcome of RA depends on the complex interaction of genetic, environmental, and host-related factors, which suggests the existence of parameters that can be evaluated to facilitate early diagnosis and prevention (Fig. 1).

Studies in monozygotic twins have estimated the relative contribution of genetic factors to be about 50 % for RA predisposition, leaving the remaining part to environmental and host issues [19]. The association between RA and major histocompatibility complex class II polymorphisms, specifically those having a conserved sequence of amino acids in a number of human leukocyte antigen DRBl alleles, called the RA-shared epitope, closely correlates with the presence of rheumatoid factor (RF), anti-citrullinated peptide antibodies (ACPA), or both [16]. This is consistent with T cell selection and antigen presentation playing a role in the induction of the autoimmune response in RA. However, the shared epitope has not been found in forms of RA negative for RF or ACPA, which may account for up to 20–30 % of patients [16, 20]. Other gene polymorphisms that differ among RA subsets include *PTPN22* (coding a tyrosine phosphatase that has a role in T-cell and B-cell signaling) [21] and genes implicated in nuclear factor κB (NF- κβ)-dependent signaling [Tumor necrosis factor receptor-associated factor 1(TRAF1-C5)] [22], interferon regulatory factors [interferon regulatory factor 5] [23], and T-cell stimulation, activation, or differentiation [signal transduction and activator of transcription 4 (STAT4)] [24].

In general, individual gene polymorphisms are of minor value for predicting disease risk unless they combine with environmental or host RA risk factors. For example, smoking is the best-defined environmental risk factor for RA, and the risk for RA increases 21-fold in smokers that are ACPA positive and homozygous for the RA shared epitope, compared to non-smokers without the shared

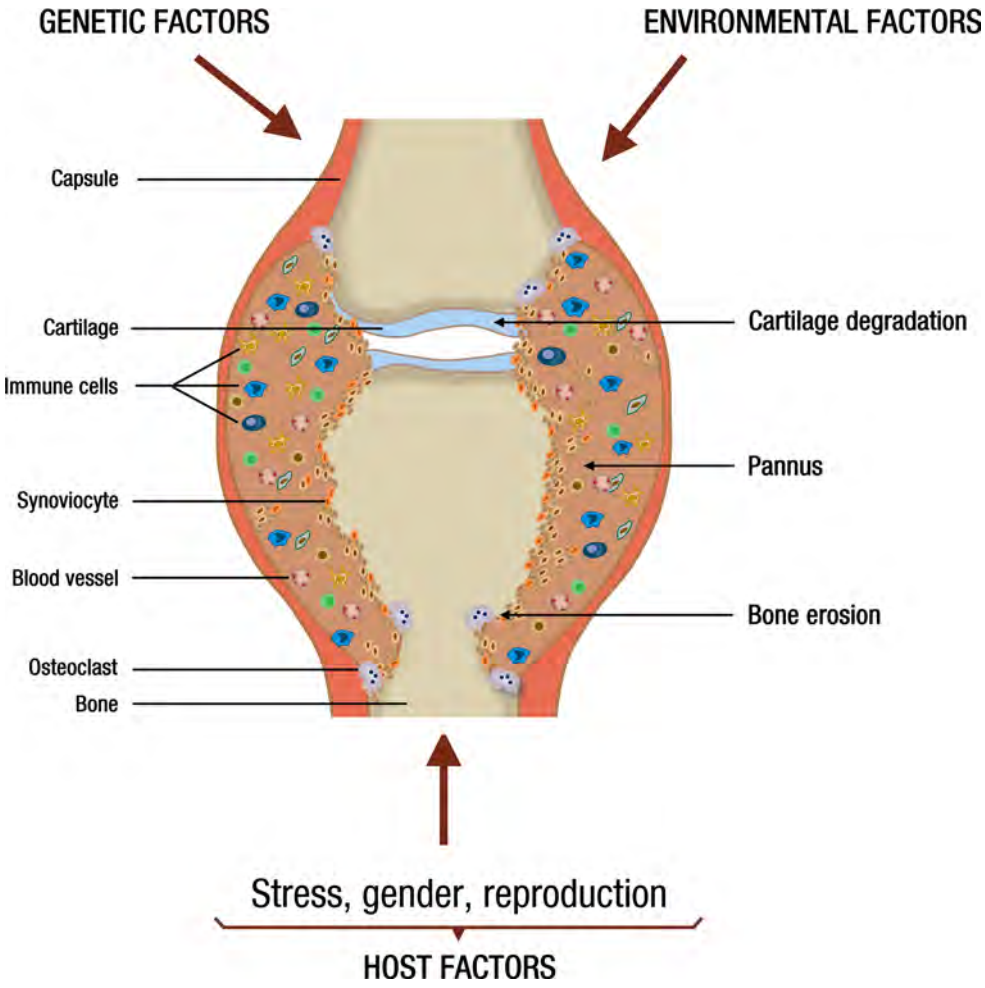

Fig. 1 Schematic view of a joint affected by RA indicating that a combination of genetic, environmental, and host factors (including stress, gender, and reproduction) influence the recruitment of immune cells and the proliferation of synovial cells, which together with the local formation of blood vessels (angiogenesis), sustain the proliferation of an inflamed, invasive pannus causing cartilage degradation and bone erosion

epitope gene [25]. Likewise, stress, gender, and reproduction are host factors influencing the susceptibility for RA in genetically restricted individuals (Fig. 1).

Stress

The long-held perception that psychological stress triggers and worsens RA has been the focus of much basic and clinical research [26–28]. Different forms of stress act in the brain (hypothalamus and brain stem) to activate efferent neuronal (i.e., norepinephrine) and hormonal (i.e., glucocorticoids, epinephrine, and PRL) pathways modifying the level of inflammation and pain [28–31]. The high prevalence of stressful events preceding the onset of RA supports the pathogenic role of stress [32], although this conclusion remains controversial [27]. Moreover, the influence of stress on RA appears to vary depending on the duration, intensity, and type of stressor, with short-lived, minor stress increasing [27, 28, 33, 34] and chronic, stronger stress ameliorating [27, 28, 35] disease activity. Similarly, exposure to different psychological or physical stressors can promote or reduce the onset and activity of experimental arthritis in rodents [36–38].

In general, stress has been considered as immunosuppressive. Exposure to stress mediators (glucocorticoids and catecholamines) counter-regulates increases in inflammatory activity by suppressing T helper 1 (Th1) cell responses and proinflammatory cytokine [interleukin-12 (IL-12), interferon γ (IFNγ), and tumor necrosis factor α (TNFα)] production and by boosting T helper 2 (Th2) responses and production of anti-inflammatory cytokines (IL-10, IL-4) [39–41]. However, glucocorticoids and catecholamines can also worsen inflammation by eliciting the expression of pro-inflammatory mediators (IL-12, IFN-γ, TNFα, IL-6, IL-23) and the down-regulation of IL-10 [28, 39, 42–44]. These opposing actions may relate to the concentration of stress hormones. There is evidence that lower cortisol, catecholamine, and PRL concentrations are immunostimulatory, whereas higher levels are immunosuppressive [26, 45–47]. Along this line, stressors can increase PRL levels in the hypothalamus by downregulating its proteolytic conversion to vasoinhibins [48], which have anxiogenic

[48] and proinflammatory effects [49]. Of note, stress axes are hyporesponsive in patients with RA [50–52] and in experimental models of RA [53], and it has been proposed that the inadequate secretion of stress hormones constitutes the basis for stress-induced aggravation of the disease [26, 28]. In this regard, elevating the levels of endogenous glucocorticoids, which appear insufficient to reduce inflammation, is the basis for their successful long-term therapeutic use in RA patients [54].

Gender and reproduction

Women are more susceptible to RA than men. Increased incidence is established before menopause, implying that hormonal and reproductive exposures are involved [55]. The sex hormones estrogens, androgens, and PRL influence susceptibility to RA. However, the metabolism of androgens to estrogens, hormone levels, age, reproductive state, and the fact that each hormone is able to exert opposing effects on immune responses have complicated the study of their role in gender- and reproduction-related RA susceptibility.

In general, androgens are anti-inflammatory and suppress both humoral and cellular immune responses, whereas estrogens reduce cellular and enhance humoral immunity [56]. Ovariectomy increases and estrogens reduce the severity of collagen-induced arthritis in female mice [57–60]. In castrated male and female arthritic rats, administration of androgens that can (testosterone) and cannot (dihydrotestosterone) be converted to estrogens alleviates the disease [61]. Because lower androgen levels occur in men [62] and in women [63] with RA, androgen deficiency could explain gender differences in RA [55, 64].

The protective role of ovarian steroids is further suggested by studies showing that contraceptive treatment decreases RA risk [65, 66] and ameliorates its severity [67], although a lack of effect has also been reported [68]. More importantly, RA often shows remission in pregnancy and exacerbation during the postpartum period in women [69] and in rodents subjected to the experimental disease [70–72]. In the context of RA being characterized by a Th1-driven immunity, the shift to a dominant Th2 phenotype occurring during pregnancy is beneficial [73], and the high gestational levels of estrogens and progesterone promote the Th2-mediated responses [74]. Therefore, protection against RA after delivery would be withdrawn by the decrease in steroid hormones and the re-establishment of a Th1 immune response [75]. Indeed, estradiol treatment immediately after parturition protects mice from a postpartum flare of the disease. Estradiol administration also increases systemic PRL, and lactating mice show a reduced postpartum exacerbation [70]. Nonetheless, breastfeeding, a stimulus that elevates circulating PRL levels, enhances the severity [76] but reduces the risk [77] of RA.

While it is evident that the influence of stress, gender, and reproduction on RA results from complex interactions between neuroendocrine and immunologic systems among persons genetically prone to RA, optimal management of such interactions could help to prevent and control the disease. More in-depth studies are needed to address the role of hormonal mediators upregulated during conditions affecting the course of RA. One such influence is the activation of the PRL/vasoinhibin axis.

PRL and vasoinhibins

PRL is a multifaceted anterior pituitary hormone discovered by its stimulatory effect on milk production; it is known to regulate a strikingly diverse array of physiological functions, which include events in reproduction, osmoregulation, growth, brain function, metabolism, immune response, and angiogenesis [3, 5, 9, 10, 13, 78]. PRL synthesis has been demonstrated in numerous extrapituitary tissues [79, 80] which, together with the ubiquitous expression of its receptors [3, 79], has led to the concept of PRL acting both as a circulating hormone and as a local regulator or cytokine. The tenet of PRL as a classical cytokine is further established by the fact that PRL is a member of the hematopoietic family of cytokines. Like other members of this family, PRL has a three-dimensional structure consisting of four long α-helices arranged in antiparallel fashion and linked by flexible loops [81]. PRL receptors also share structural and functional characteristics with the hematopoietin receptor superfamily [81], and they signal by activating various kinases including its canonical Janus kinase 2 (JAK2)-signal transducer and activator of transcription (STAT) pathway, the phosphatidylinositol 3-kinase (PI3K)/AKT pathway, and the mitogen-activated protein kinase (MAPK) pathway [82].

Adding complexity to PRL actions is the structural polymorphism of the hormone [83]. PRL is proteolytically cleaved to vasoinhibins, a family of PRL fragments with molecular masses ranging from 11 to 18 kDa, that signal through receptor complexes distinct from the PRL receptor [84, 85] to exert effects opposite to those of the full-length hormone (23 kDa) on blood vessels. PRL stimulates blood vessel growth (angiogenesis), whereas vasoinhibins inhibit angiogenesis, vasodilation, and vasopermeability [9, 13]. Beside vascular actions, vasoinhibins have other effects opposite to PRL. Vasoinhibins promote anxiety [48] whereas PRL is anxiolytic [86]; and vasoinhibins [49] and PRL [87] stimulate and inhibit inflammatory reactions in lung tissues, respectively. The structural diversity of vasoinhibins derives from the fact that different proteases,

including cathepsin D, matrix metalloproteases (MMP) and bone morphogenetic protein-1 (BMP-1), generate the various fragments by cleaving near or within various sites of the large disulfide loop linking α-helixes 3 and 4 of the PRL molecule (for reviews [13, 14, 88]). Of relevance to RA, joint tissues are targets of the PRL/vasoinhibin axis. PRL is present in synovial fluid [89], and both PRL and vasoinhibins are produced in joint tissues, including cartilage [90], vascular endothelium [91], synoviocytes [92], fibroblasts [49], and immune cells [10, 79, 92], where they can act in local- and hormonal-related fashion.

Effects of PRL and vasoinhibins on joint tissues

A summary of PRL and vasoinhibin effects on joint tissues is illustrated in Fig. 2.

Bone and cartilage

Bone loss occurs in association with hyperprolactinemia during lactation, in patients with prolactinomas, and in patients treated with antipsychotic drugs [93–96]. Although increased bone loss involves PRL-induced hypogonadism [93, 97] and the interaction of PRL with other hormones (parathyroid hormone related peptide [98]), clinical [96] and experimental evidence support direct effects of PRL on bone metabolism. PRL receptor null mice are osteopenic and display a reduced rate of bone formation [8], indicating that PRL promotes the maintenance of bone mass. However, PRL has opposite effects on bone depending on the age and physiological state of the animals. Prolonged treatment with PRL increases bone formation and bone calcium content in growing rats [99], whereas it stimulates bone resorption and calcium turnover in sexually mature rats [100] and in pregnant and lactating rats [101]. In the latter, bromocriptine-induced inhibition of PRL secretion results in higher bone volume, lower mineral apposition rates, and reduced bone resorption [102]. It has been proposed that PRL stimulates maternal bone turnover during pregnancy and lactation as a means to satisfy the demands of calcium and phosphate needed for fetal growth and milk production [102].

Of note, PRL can act directly on bone. PRL receptors have been found in bone tissues of humans, mice, and rats [103, 104] and in osteoblasts from neonatal rodents [8, 105] and human fetuses [106]. Also, high levels of PRL stimulate bone resorption over bone formation in cultured osteoblasts derived from adult tissues. In these cells, PRL reduces the expression of Runx2 and osteocalcin, lowers alkaline phosphatase activity, and increases the ratio of receptor activator of nuclear factor κB ligand (RANKL) to osteoprotegerin [106, 107]. In contrast to these findings and consistent with the net bone gain effect of PRL observed in

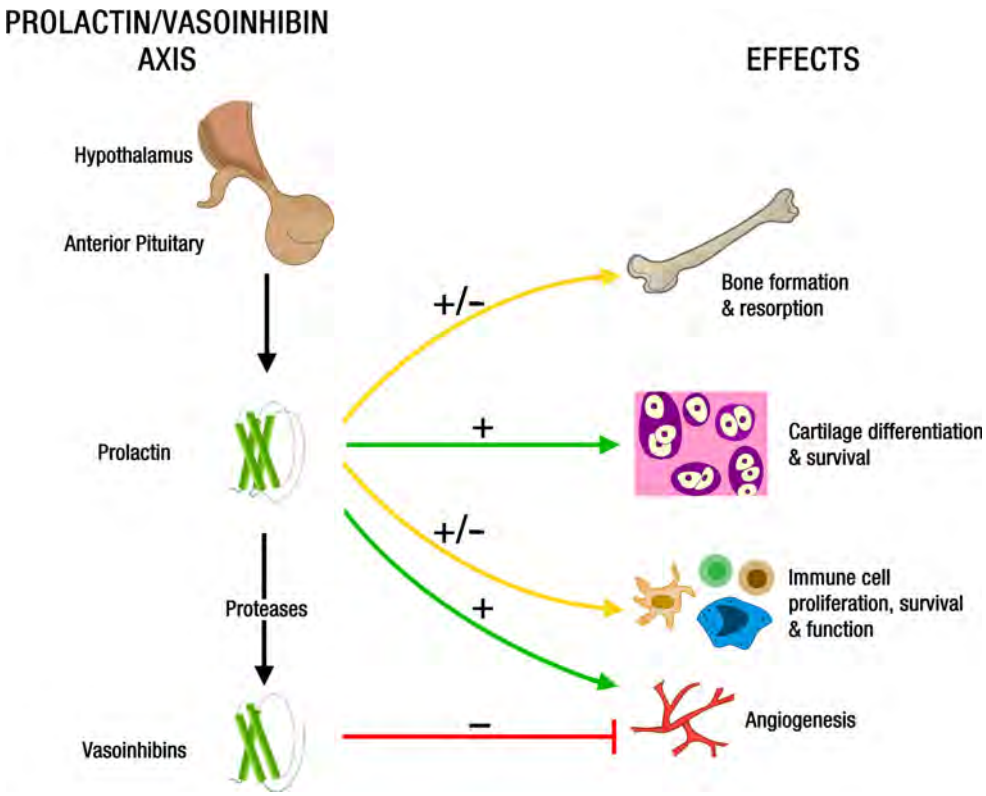

Fig. 2 Schematic representation of PRL and vasoinhibin effects on joint tissues. The anterior pituitary gland secretes PRL and vasoinhibins into the systemic circulation. Also, PRL produced locally by joint tissues can be proteolytically converted to Vi. By virtue of mechanisms poorly understood, PRL stimulates and inhibits (±) bone formation and resorption, promotes (+) cartilage differentiation and survival, and stimulates and inhibits (±) immune cell proliferation, survival, and function. In addition, PRL is proangiogenic (+) and, upon proteolytic cleavage to vasoinhibins, acquires antiangiogenic (−) properties. The effects of Vi on bone, cartilage, and immune cells are unknown

young animals, studies on PRL-exposed osteoblasts from fetal bone showed enhanced Runx2 and osteocalcin expression and a decreased RANKL/osteoprotegerin ratio, suggesting stimulation of bone formation and suppression of bone resorption, respectively [106, 108].

Bone tissues affected by PRL include cartilage. Chondrocytes in both reserve and proliferating regions of the developing long bone express PRL receptors [105], but cartilage formation and growth do not seem to be adversely affected in the PRL receptor null mice [8] or after elevating PRL levels during early life [105]. Nonetheless, PRL may contribute to the chondrogenic process by regulating cartilage formation and maintenance in the adult. Human adult bone marrow pluripotential mesenchymal stem cells express PRL receptors and PRL stimulates their proliferation and differentiation into chondrocytes (type II collagen and proteoglycan synthesis) in culture [89]. Also, PRL activates their receptors in articular chondrocytes from rat postpubescent and adult cartilage to inhibit apoptosis [7]. Because cartilage lacks blood vessels, the sparse distribution of chondrocytes encased within the extracellular matrix suggests that autocrine/paracrine anti-apoptotic factors are an efficient mechanism for maintaining cartilage survival. Along this line, PRL is a component of synovial fluid [89] and is generated by articular chondrocytes [90] and bone-marrow mesenchymal cells undergoing chondrogenic differentiation [89]. Therefore, PRL may help preserve the generation and functional integrity of cartilage. Moreover, chondrocytes produce several MMP that generate vasoinhibins from circulating and locally produced PRL and release vasoinhibins into their conditioned medium [90], suggesting that the PRL/vasoinhibin axis contributes to the local maintenance of cartilage avascularity.

Blood vessels

The regulation of blood vessel growth and function underlies the physiology of joint tissues. Fenestrated capillaries close to the synovial surface are a main source of synovial fluid, a plasma ultra-filtrate transporting lubricants, nutrients, waste products, hormones, growth factors, and cytokines within the joint, particularly to the avascular cartilage. Avascularity is essential for the biomechanical properties of cartilage and, although the subchondral vasculature contributes to the metabolic support of normal articular cartilage [109], vascular invasion from bone into cartilage leads to chondrocyte apoptosis and is a mechanism mediating cartilage substitution by bone during growth [110]. While blood vessels are known targets of PRL and vasoinhibins, and chondrocytes produce these peptides [90], no study has addressed the actions of the PRL/vasoinhibin axis on the joint vasculature.

The effects and signaling mechanisms of PRL and vasoinhibins on blood vessels have been well documented and reviewed [9, 13, 88]. The following summarizes some of the relevant findings. PRL promotes in vivo angiogenesis in the corpus luteum [111], the testis [112], the heart [113], the pancreas [114], and the liver [115]. This action involves a direct effect on endothelial cells [116–118], through both long and short isoforms of the PRL receptor [119–121], but also through the PRL receptor on other cell types (epithelial, immune, and stromal cells) where PRL induces the expression of proangiogenic factors, such as vascular endothelial growth factor (VEGF) [122, 123] and fibroblast growth factor-2 (FGF-2) [124, 125]. PRL signals to promote endothelial cell proliferation, migration, and tube formation through the JAK2/MAPK/early growth response gene-1 pathway [122], the heme oxygenase-1 (HO-1) [116], and the JAK2/STAT5 pathways [118]. However, controversial findings (for a review see [9, 13, 126]) have shown that PRL is unable to stimulate angiogenesis in the cornea and the in vitro proliferation of some endothelial cell types. Also, lack of PRL expression increases angiogenesis in the retina [127] and associates with highly vascularized pituitary tumors [128]. Moreover, PRL has opposing effects on vascular resistance, blood flow, and vasopermeability that depend on experimental conditions (for a review see [126]). These inconsistencies may result from a variable rate of proteolytic conversion of PRL to vasoinhibins.

Vasoinhibins inhibit vasoproliferation, vasodilation, and vasopermeability and promote vascular regression in the cornea [129], retina [127, 130], heart [113], and xenografted tumors [85]. They act directly on endothelial cells to inhibit the action of several vasoactive substances, including VEGF, FGF-2, interleukin 1β, bradykinin, and acetylcholine [130–135]. Binding sites for vasoinhibins were reported in endothelial cell membranes, but their chemical nature was not resolved [84]. Recently, vasoinhibins were shown to bind to a multicomponent complex conformed by plasminogen activator inhibitor-1 (PAI-1), urokinase plasminogen activator (uPA), and the urokinase plasminogen activator receptor (uPAR) on endothelial cells and that such binding was required for some antiangiogenic properties of vasoinhibins [85]. Vasoinhibins signal by blocking the activation of the Ras–Raf–MAPK pathway [136, 137], the Ras–Tiam1–Rac1–Pak1 pathway [133], and the Ca^{2+}/calmodulin-mediated activation of endothelial nitric oxide synthase (eNOS) [134]. They also promote protein phosphatase 2A-induced dephosphorylation and inactivation of eNOS [130], downregulate transient receptor potential channel 5 expression [138], stimulate the activation of proapototic proteins of the Bcl-2 family, and the NFκB-mediated activation of caspases [139] and upregulation of microRNA-146a expression [140].

Therefore, PRL and vasoinhibins act through the activation of distinct receptors and target cells to stimulate and inhibit blood vessels, respectively. Vasoinhibins bind to a multi-component complex involving PAI-1, uPA, and uPAR on endothelial cells, while PRL signals through the PRL receptor not only on endothelial cells, but also on other cells in the capillary microenvironment, including immune cells (Fig. 2).

Immune cells

The action of PRL on immunocytes has been known for more than three decades, when PRL effects [141] and PRL receptors [142] on immune cells were first discovered. PRL operates not only by systemic but also by paracrine/autocrine mechanisms to regulate immune responses [10–12, 29, 80, 143–145]. PRL and the PRL receptor (PRLR) are expressed on T cells, B cells, natural killer (NK) cells, macrophages, neutrophils, and antigen-presenting dendritic cells; and PRL regulates various immune cell responses, including T-cell proliferation and survival, B-cell antibody production, NK-cell proliferation and mediated cytotoxicity, and macrophage effector functions. Notably, PRL exhibits both inmunoenhancing and immunosuppressive properties depending on its concentration (with lower levels augmenting and higher levels inhibiting immune responses) [12, 143, 145].

The first observation linking high PRL levels with immunosuppression was made more than 40 years ago, when lactation [146], as well as PRL treatment [147], was shown to increase susceptibility to worm-infection resulting from impaired differentiation of lymphocytes to mature effector cells. The opposite actions of different PRL concentrations was initially suggested by showing that a lower dose of PRL (100 μg/rat) stimulated antibody production and T cell proliferation, whereas a higher dose (400 μg/rat) was inhibitory [148]. Also, the number and function of circulating NK cells and mature T cells were reduced in patients with hyperprolactinemia (32–394 ng/ml) [149–151], and in vitro studies showed that higher (100–200 ng/ml) and lower (12–25 ng/ml) PRL levels inhibit and stimulate, respectively, the proliferation of blood NK cells and T cells [47]. Likewise, lower concentrations of PRL (<20 ng/ml) were more effective than higher PRL levels (100 ng/ml) in stimulating antibody production by circulating lymphocytes from patients with systemic lupus erythematosus [152]; while high doses of PRL enhanced the in vitro release of proinflammatory mediators by peritoneal macrophages and peripheral blood mononuclear cells, a much higher dose induced the release of anti-inflammatory IL-10 [153, 154].

Nevertheless, it has been widely assumed that PRL functions as an immuno-enhancing hormone. This notion is largely based on the use of experimental models of PRL deficit, including dwarf mice [155], hypophysectomized rats [144], and treatment with bromocriptine (a dopamine D2 receptor agonist that inhibits pituitary PRL release) in rodents and humans [11, 144]. These experimental approaches result in deficient B- and T-cell-mediated immune responses that can be restored by PRL treatment. However, it should be noted that multiple endocrine pathways are being altered in these experimental models, PRL is also produced by extrapituitary sources [79, 80], and bromocriptine can affect immune function independently of PRL [156, 157]. A more direct evaluation of loss of PRL function on the immune system has been done using PRL [158] and PRLR [159] null mice. Both mice exhibit normal numbers of lymphocytes and myeloid cells and mount effective innate and adaptive immune responses when subjected to immune challenges, thus implying that PRL is not essential for normal immune system development and function. Nonetheless, compensatory actions by other cytokines have not been examined, and experiments using these mice have not ruled out a contribution of PRL to immune-system homeostasis under conditions characterized by the up-regulation of PRL levels, such as under stress [29], reproductive adaptations, and pathologic hyperprolactinemia [160].

Indeed, renewed interest has focused on the possibility that elevated circulating PRL levels following stress [1, 2] represent an adaptation to help adjust the frequently observed stress-related immunosuppression [29]. PRL improves macrophage and splenocyte functions following trauma-hemorrhage and infections [161, 162], stimulates lymph node cellularity and antigen-specific proliferative responses under stressful housing conditions [155, 163], and it can help counteract glucocorticoid-induced lymphocyte apoptosis and signaling [164, 165]. However, PRL can also inhibit immune responses during stress. Bone marrow myelopoiesis and splenic lymphocyte proliferation induced by a burn injury are enhanced in PRL null mice [166], PRLR knockout mice show increased mortality and elevated IL-6 after partial hepatectomy [115], and PRL administration associates with decreased survival and an inhibition of cellular immune functions in septic mice [167]. The reason for the opposite effects of PRL on immune function during stress is unclear, but may relate to differences in PRL levels and in immune processes occurring in response to specific stressors. A possible explanation also involves the generation of vasoinhibins from PRL. Exposure to physical restraint increased PRL levels in the hypothalamus partly by reducing its rate of cleavage to

vasoinhibins [48]. Because vasoinhibins are anxiogenic, this change would favor the anxiolytic effect of PRL, thereby reducing the emotional response to stress [48]. Similarly, such reciprocal interplay during stress could favor PRL-induced suppression of inflammatory responses. Vasoinhibins act as potent proinflammatory cytokines, via the activation of NFκB signaling pathways, to stimulate inducible nitric oxide synthase (iNOS) expression and nitric oxide (NO) production in fibroblasts and type II epithelial cells of the lung [49, 168], which are important cells for inflammatory reactions in the airways. Conversely, PRL has anti-inflammatory effects on the same cells [87].

Pregnancy and lactation constitute other important contexts for understanding the role of PRL in the immune system. Elevated PRL levels in pregnancy and early stages of lactation may help suppress immune responses required for successful maternal-fetal and maternal-nursing young interactions. Excessive production of IL-6 in the decidua can trigger an inflammatory response that leads to the termination of pregnancy, and estradiol and PRL inhibit IL-6 expression and signaling by decidual cells [169]. Moreover, PRL produced by the decidua [170] and PRL-like proteins produced by the placenta [171] interact with maternal immune cells (NK cells and megakaryocytes) to ensure successful fetal development [172, 173], and PRL regulation of humoral immunity can influence the levels of immunoglobulins in milk [174–176].

PRL and RA

The association of RA with stress, gender, and reproduction prompted the investigation of PRL as a biological explanation, but the significance of PRL to RA pathogenesis remains unclear. Several reviews have addressed this topic under the general assumption that PRL is a negative (proinflammatory) influence aggravating the disease [10, 177–186]. This conclusion contrasts with the lack of agreement between circulating PRL levels and disease severity, the inconsistent action of dopamine agonists and antagonists, the protective and worsening influence of physiological hyperprolactinemia (pregnancy and lactation), and the ability of PRL to exert both stimulatory and inhibitory effects on immune cells and joint tissues. Our work reviews this controversial field with the idea that its solution requires understanding the mechanisms mediating the dichotomous actions of PRL. Because PRL and vasoinhibins have opposite effects on immune reactions and angiogenesis, we propose that explanatory mechanisms lie within the PRL/vasoinhibin axis, an integrative framework influencing not only the levels of systemic and local PRL, but also the proteolytic conversion of PRL to vasoinhibins.

Clinical data

Several [182, 187–189], albeit not all [189–191], epidemiological studies have claimed that nulliparity and, paradoxically, the postpartum period increase the risk for developing RA. Nulliparity could be linked to hyperprolactinemia-induced infertility [97] and, of course, breastfeeding in the postpartum period elicits hyperprolactinemia. Originally, the connection between breastfeeding and RA generated controversial findings. Exposure to breastfeeding associated with increased risk [192, 193], had no effect [189] or was protective [194] against RA development. However, large cohort studies have now shown that breastfeeding for more than 12 months is inversely related to the development of RA and suggested that the protective effect of parity could be attributed to confounding breastfeeding in previous lactations among parous women [77, 191, 195].

The associations above encouraged an investigation of PRL levels in the circulation of patients with RA (Table 1). Several reports found that PRL levels were higher, albeit usually within the normal range (≤20 ng/ml), in patients with RA compared to healthy or osteoarthritic subjects [196–204]. Nonetheless, no differences [51, 52, 205–209], as well as lower than normal levels of the systemic hormone [210], have also been reported in RA. Because PRL levels vary according to the time of day and stress exposure [1, 2, 211], studies attempting to control for these variations showed that the RA group had significantly higher PRL levels at the diurnal hormonal peak, before and after stress (surgery), and following thyrotrophin releasing hormone (TRH) stimulation [197, 202]. However, no difference in PRL levels after TRH or even a decreased PRL release in response to other forms of stress (hypoglycemia), were also found [51, 205, 209]. Moreover, lowering circulating levels of PRL with dopamine D2 receptor agonists (bromocriptine, quinagolide, cabergoline) proved to be effective [177, 212–214], or ineffective [215–217] for reducing RA. In one report, RA improved after treatment with cabergoline in a hyperprolactinemic patient (76.5 ng/ml) with a microprolactinoma [214]. However, cases of PRL excess due to prolactinomas appear to be extremely rare in autoimmune diseases and are not consistent with their severity [218]. Also, PRL levels above normal have been detected in only 1 or 6 % of patients with RA and have either no correlation with disease activity [219] or a low disease activity score [220]. Along this line, human pathological hyperprolactinemia has been associated with immunosuppression (decreased number and function of NK cells and T cells) [149–151].

The major endpoint of these studies is the concentration of systemic PRL. However, it is now clear that local PRL produced and metabolized in the tissues can be influential.

Table 1 Circulating PRL levels in RA

PRL levels	RA (*n*)	Control (*n*)	Serum PRL (ng/ml)		*p* value	Special features	References
			RA	Control			
Higher	99	68 (OA)	11.7 ± 7.6[a]	8.9 ± 4.0	<0.01	PRL levels correlate with RA duration and severity	[196]
	10	10 (OA)	21.2–26.9[a]	4.7–20.3	<0.005	Higher PRL diurnal levels and after surgery in RA	[197]
	39	22	16.0 ± 11.2[a]	10.4 ± 6.9	<0.05	PRL levels correlate with chemokine MIP-1α serum levels	[198]
	29	30 (OA)	13.1 ± 1.7[b]	7.5 ± 0.5	<0.01	PRL levels correlate with RA duration	[199]
	10	10	20.6 ± 5.1	12.1 ± 4.3	<0.005	Higher PRL levels in RA only at 0200 h	[200]
	29	26 (OA)	14.1 ± 1.3[a]	10.9 ± 0.8	<0.04	PRL levels correlate with RA severity	[201]
	23	8	27.3 ± 3.8[b]	8.8 ± 2.2	<0.01	Higher PRL levels in response to TRH	[202]
	60	31 (OA)	10.6 ± 4.9[a]	8.3 ± 3.2	<0.04	Both total and *free PRL were measured	[203]
	20	20	40.2 ± 5.6[a]	16.2 ± 3.1	<0.001	PRL levels correlate with ESR and C-reactive protein	[204]
Not different	38	23	10	8	n.s.	Higher and lower PRL levels in response to hypoglycemia and TRH, respectively	[205]
	27	12 (OA)	6.05 ± 1.1[b]	8.49 ± 2.4	n.s.	PRL levels in plasma and synovial fluid correlate positively	[206]
	20	28	11.5 ± 7.4[a]	12.5 ± 6.5	n.s.	No association with thyroid autoantibodies in RA	[207]
	10	10	10.1 ± 1.4[b]	13.7 ± 2.4	0.32	Similar PRL levels in response to TRH between RA and control	[208]
	10	9	10	12.5	n.s.	Similar PRL levels in response to TRH between RA and control	[209]
	20	20	13.0 ± 7.5[a]	15.0 ± 8.0	0.2	Reduced PRL levels in response to hypoglycemia in RA	[51]
	7	10	10.6	10.3	n.s.	Lower increase in PRL levels in response to exercise in RA vs control	[52]
Lower	48	23	7.9 ± 0.3[b]	9.5 ± 0.5	<0.05	Association between RA and PRL deficiency	[210]

In some studies PRL data were converted from units to mass (ng/mL × 21.2 = mIU/L). OA indicates osteoarthritic patients as control group *MIP-1α* macrophage inflammatory protein-1α, *TRH* thyrotropin releasing hormone, *ESR* erythrocyte sedimentation rate, *ns* non-significant

PRL values are [a] means ± SD, [b] mean ± SEM, or means

* Free PRL refers to monomeric, unbound PRL

In RA, synovium infiltrating T lymphocytes and fibroblast-like synovial cells produce PRL able to promote inflammation at the local level [92]. PRL stimulates proliferation and the production of proinflammatory cytokines and MMP, and it inhibits the synthesis of tissue inhibitor of MMP (TIMP-I) by RA synovial cells in culture [92]. Moreover, bromocriptine inhibits the expression of PRL mRNA by primary cultures of RA synovial cells [92] and can suppress their activity in a PRL-dependent and PRL-independent manner [92, 156, 157]. Also, the presence of a PRL-1149 T polymorphism in the promoter region controlling the production of extrapituitary PRL associates with reduced PRL production by lymphocytes [221], but not with the systemic levels of the hormone, and correlates with a decreased risk of developing RA [222, 223]. Therefore, local PRL levels may correlate better than systemic PRL levels with the ongoing inflammation.

An additional complexity is added by the paradox that activation of dopamine D2 receptors may also protect against RA. Haloperidol, a D2-receptor antagonist leading to hyperprolactinemia [224], alleviates inflammation in RA [225] by mechanisms that may include PRL-induced immunosuppression [6] and the direct blockage of D2 receptors on immune cells able to reduce proinflammatory cytokine release and action [226, 227].

The fact that both dopamine agonists and antagonists ameliorate RA may be due to the negative and positive effects of PRL on immune cells and joint tissues. To understand these opposite actions, it is important to study how PRL levels in the circulation and within the tissue microenvironment are being regulated to affect the function of the various targets. Experimental studies exploring the role of PRL in inflammatory arthritis mirror these conflicting observations but have also contributed to their clarification.

Experimental data

The causative link between PRL and RA is supported by studies using complete Freund's adjuvant (CFA)-induced arthritis in rats, a well-documented model for the induction of inflammation within joint tissues and for having cartilage and bone destruction similar to that in RA [228, 229]. Early studies claimed that the development of adjuvant arthritis was at least partially dependent on PRL. Hypophysectomized rats did not develop CFA arthritis

unless treated with PRL, and the CFA-induced joint swelling in intact rats was reduced by treatment with bromocriptine [230]. Moreover, hypophysectomized rats made hyperprolactinemic by placing anterior pituitary glands (AP) under the kidney capsule developed a more severe arthritis than sham-operated controls [230, 231]. It was reasoned that adrenocortical deficiency due to hypophysectomy favored the manifestation of PRL proinflammatory effects [231]. However, hypophysectomy could be a confounding factor. AP grafts and high PRL levels in the intact organism stimulate glucocorticoid release [232], and APs grafted into sham-operated rats result in a delay in the onset and a reduction in the severity of CFA-induced arthritis and higher corticosterone circulating levels [231]. Therefore, adrenocortical dysfunction due to hypophysectomy could also be counterbalancing the beneficial effects of PRL.

Recent findings support the protective and regenerative effects of hyperprolactinemia in CFA-induced arthritis [6]. Increasing prolactinemia, either by PRL infusion or treatment with haloperidol, before or after inducing arthritis with CFA, ameliorated inflammation and joint destruction as revealed by lower proinflammatory cytokine (TNFα, IL-1β, IFNγ, IL-6) and iNOS expression in joint tissues, and reduced chondrocyte apoptosis, pannus formation, bone erosion, joint swelling, and pain. Proinflammatory cytokines are crucial for initiating the inflammatory process leading to joint destruction in RA [233, 234]. The concentration and expression of proinflammatory cytokines are significantly elevated in serum [228] and joint tissues [6] of CFA-injected rats, respectively, and IL-1 antagonists and TNFα-neutralizing antibodies reduce the severity of arthritis in these animals [229, 235]. Accordingly, PRL could be protecting against CFA-induced arthritis not only by reducing the levels of proinflammatory cytokines, but also by counteracting their proapoptotic and inflammatory effects. PRL inhibited the apoptosis of cultured chondrocytes in response to a combination of TNFα, IL-1β, and IFNγ by blocking the induction of p53 and decreasing the BAX/BCL-2 ratio through a NO-independent, JAK2/STAT3-dependent pathway [6]. Local treatment with PRL or increasing PRL circulating levels also prevented chondrocyte apoptosis evoked by injecting the cytokines into the knee joints of rats, whereas the proapoptotic effect of cytokines was enhanced in PRL receptor null mice [6]. Similar findings were also reported in pulmonary fibroblasts, where the induction of iNOS expression and NO production in response to TNFα, IL-1β, and IFNγ was reduced by PRL [87].

However, other studies have argued against the anti-inflammatory effect of PRL in arthritis. PRL treatment enhances disease progression of collagen-induced arthritis only when delivered during the immunization phase [236], although the same report revealed that treatment with bromocriptine caused exacerbation at a later stage of the disease. In addition, PRL enhances the proliferation and release of IL-6, IL-8, and MMP-3 by RA synovial cells in culture and lowers their production of TIMP-1 [92]. While variations in experimental conditions may contribute to these discrepancies, it cannot be disregarded that PRL can have both protective and exacerbating effects on arthritis and that addressing this paradox is challenging.

Opposing effects may relate to PRL levels since, as previously described, low doses can be proinflammatory and higher doses anti-inflammatory [47, 148, 152]. PRL levels are affected by exposure to a variety of active substances and hormones in the inflammatory milieu. There are reports showing that CFA-induced arthritis lowers [237, 238], enhances [239], or has no effect [6, 240] on PRL circulating levels. Proinflammatory cytokines, such as TNFα and IL-6, stimulate the release of PRL by AP cells [241, 242], and IL-1β, IL-2, and IL-4 downregulate PRL expression in immune cells [243].

Importantly, the inflammatory milieu could influence the conversion of PRL to vasoinhibins. MMP are upregulated in the joints of patients with RA [244] and proinflammatory cytokines [233] and PRL [92] stimulate MMP production by RA synovial cells. MMP cleave PRL to vasoinhibins [90] which, by being antiangiogenic, are able to suppress the neovascularization required for pannus formation [245]. Angiogenesis occurs from the early stage of RA and supports the invasive pannus by enabling the continued accumulation of immune cells, the proliferation of the inflamed tissue, and the swelling of the joints [246, 247]. The upregulation of MMP in the arthritic joint (in response to cytokines including PRL) and the fact that hyperprolactinemia reduces pannus formation [6] raise the possibility that, under a sustained administration of PRL, there is an enhanced generation of vasoinhibins which, by virtue of their antiangiogenic properties, contribute to the protective and regenerative effects of PRL.

The above proposal is challenged by the fact that increased collagenase activity leads to joint destruction and is the basis for the use of MMP inhibitors in RA clinical trials [248]. There is also evidence that vasoinhibins act as potent proinflammatory cytokines in lung tissues [49, 168], where PRL is anti-inflammatory [87, 249]. Much work is needed to characterize the proinflammatory effects of vasoinhibins, and nothing is known regarding the actions of these peptides in joint tissues. However, the notion that full-length PRL has anti-inflammatory and proangiogenic effects and acquires pro-inflammatory and antiangiogenic properties after undergoing proteolytic cleavage to vasoinhibins provides an efficient mechanism for balancing these two processes and a new avenue that may clarify the controversial role of this hormone in pathologies such as

RA that are characterized by inflammation and exacerbated angiogenesis.

Enhanced RA risk and severity correlate with pregnancy complications affecting the functionality of the PRL/vasoinhibin axis, which may help illustrate the considerations above. Such adverse pregnancy outcomes include preterm and small-for-gestational-age delivery [250], delivery of infants with very low and extremely low birth weight [251], low birth weight in general [252], and preeclampsia [253]. The levels of vasoinhibins are elevated in the serum, urine, and amniotic fluid of patients with preeclampsia, and vasoinhibin values in amniotic fluid are inversely correlated with birth weight [254–256]. Excessive inflammation and dysregulation of angiogenesis inhibitors are key factors in the etiology of preeclampsia [257] that may be influenced by an imbalance of the PRL/vasoinhibin axis [254, 258] and lead to the increased risk and severity of RA.

Evolutionary implications

RA belongs to a large family of acute and chronic arthritic diseases defined as inflammatory arthropathies that, in humans, also include spondyloarthritis (comprising ankylosing spondylitis, reactive arthritis, psoriatic arthritis, arthritis conditions associated with inflammatory bowel disease), septic arthritis, gout, and osteoarthritis. These are ancient diseases with cases identified in fossil records from millions of years ago in Triassic dinosaurs and Pleistocene mammals, and from thousands of years ago in Neolithic man (reviewed by [259]). In spite of evolutionary pressures, inflammatory arthropathies stand as prevalent disorders across contemporary terrestrial vertebrates. To explain such prevalence, it is proposed that the painful and limiting characteristics of arthritis could have represented an adaptation selected to restrain animals from physically demanding activities and energy expenditure when facing adverse conditions such as starvation, stress, pregnancy, lactation, and aging [260]. The strong association between inflammatory arthropathies and age, gender, and reproduction suggests that factors upregulated under these conditions helped to maintain these diseases throughout evolution. One such factor is PRL, a stress-related, sexually dimorphic, reproductive hormone with an ancient origin. The reader is directed to a recent overview addressing this idea [259].

PRL is thought to have evolved 400 millions years ago [261] to regulate osmoregulation and dispersion of skin pigments in fish and amphibians, ancestral activities that continue to be present in the PRLs of higher vertebrates [262]. Likewise, providing nutrients to the young and stimulating parental behavior, the best-known effects of PRL, also emerged in fish and have been retained in fish, birds, and mammals [259, 262].

The high-energy demands associated with feeding and tending the young make wild organisms particularly susceptible to adverse stressful conditions. Stress downregulates PRL levels in birds, thereby interfering with parental behavior [263]. In mammals, there is an attenuation of physiological and behavioral stress responses during pregnancy and lactation [264] that is counteracted by inhibiting the expression of PRL receptors in the brain [86]. Reduced reaction to stress not only ensures maternal care under aversive conditions, but can also be detrimental for parent survival. PRL inhibits anxiety [86] but acquires anxiogenic properties upon conversion to vasoinhibins [48]; thus, the PRL/vasoinhibin axis [14] may represent an efficient mechanism for adjusting stress responses and maximizing reproduction and survival under challenging conditions such as arthritis.

While ample evidence exists in rodents and humans, there have been remarkably few or no studies in non-mammalian vertebrates regarding effects of PRL on joint tissues and immune cells. However, the facts that reproductive effects of PRL and PRL stress-related interactions are phylogenetically conserved and that PRL receptors are present in immune, bone, and muscle cells of fish [265, 266] and birds [267, 268] prompt the speculation that the PRL functions in non-mammalian vertebrates, similar to its role in mammals, as an epigenetic, adaptive regulator of arthritis development, influencing its prevalence throughout evolution.

As for the PRL/vasoinhibin axis, a PRL sequence comparison between species of several taxons indicates the possibility that vasoinhibins first emerged in tetrapods as no vasoinhibin-generating cleavage site was identified in Teleost fish (Zebrafish), Ray-finned-fish (Spotted gar), and Lobe-finned fish (Coelacanth). Amphibians, reptiles and birds, however, possess cleavage sites required for the generation of vasoinhibins, indicating the possible existence of a PRL/vasoinhibin axis in these vertebrates [14]. The emergence of the PRL/vasoinhibin axis in tetrapods correlates well with the effects of this axis on joint physiology and disease, as arthritis essentially occurs in terrestrial vertebrates [259].

Concluding remarks

The influence of stress, gender, and reproduction on the pathogenesis of RA points to a possible contribution of PRL, a stress-related, sexually dimorphic, reproductive hormone with effects on joint tissues and immune cells. The female preponderance of RA, the increased levels of PRL in the circulation of patients with RA, and the ability

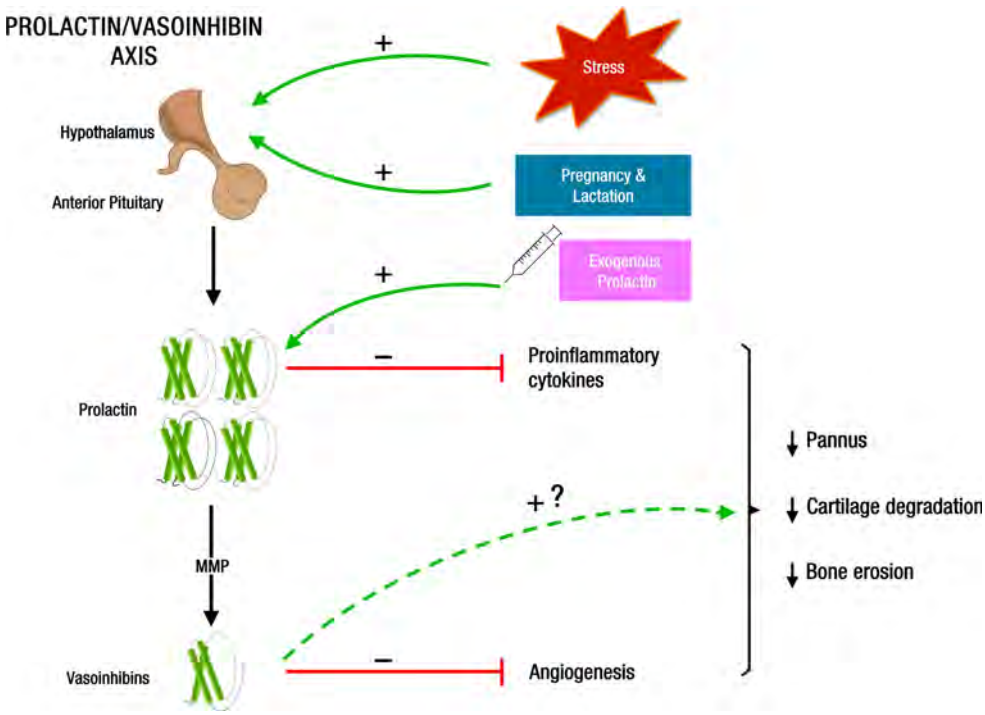

Fig. 3 Mechanisms that contribute to PRL-mediated protection against RA. Hyperprolactinemia induced by stress, reproduction (pregnancy and lactation), and PRL administration acts on joint tissues to reduce proinflammatory cytokine expression and action and to upregulate PRL cleaving proteases (MMP) leading to vasoinhibin-mediated antiangiogenesis; these effects, in turn, lead to reduced pannus formation, cartilage degradation, and bone erosion. Vasoinhibins may act as proinflammatory factors able to counteract PRL anti-inflammatory effects in arthritis, a possibility that needs to be investigated

of PRL to promote inflammation have been the basis for the long-held notion that this hormone is a negative factor aggravating RA. However, the risk and severity of RA are downregulated in reproductive states characterized by hyperprolactinemia (pregnancy and lactation); the circulating levels of PRL only increase in a small proportion of RA patients, and this hormone is also immunosuppressive. The paradox of PRL having both protective and exacerbating effects in RA is better explained when considering the PRL/vasoinhibin axis. This organizational principle takes into account that PRL actions are regulated at the hypothalamus, pituitary gland, and target-tissue levels not only by altering the synthesis and release of systemic and local PRL, but also by regulating the proteolytic conversion of PRL to vasoinhibins as PRL and vasoinhibins activate distinct receptor complexes and cell types to exert their opposite effects on blood vessels and inflammatory reactions. The PRL/vasoinhibin axis is exposed to a variety of active substances that vary according to stress-related and reproductive events preceding and modifying the progression of RA. Some of these agents (catecholamines, glucocorticoids, sex steroids, proinflammatory cytokines, anti-inflammatory cytokines) alter the systemic and local concentrations of PRL and vasoinhibins. Variations in the levels of PRL and vasoinhibins influence the direction of their action in RA. Lower and higher levels of PRL are immunostimulatory and immunosuppressive, respectively, and vasoinhibins counteract the anti-inflammatory and proangiogenic effects of PRL. Of much interest are observations in which hyperprolactinemic states (stress, pregnancy, and lactation) and PRL treatment protect against inflammation and joint destruction in arthritis by mechanisms that may include reduced proinflammatory cytokine expression and action and the upregulation of cleaving proteases (MMP) leading to vasoinhibin-mediated antiangiogenesis, actions that lead to reduced pannus formation, cartilage degradation, and bone erosion (Fig. 3). A therapy based on elevating PRL serum levels may be comparable to the well-established use of glucocorticoids in patients with RA, whose levels of the endogenous hormones appear insufficient to control the disease [269].

There is much to learn about the mechanisms mediating the opposite effects of PRL on joint tissues and immune cells and about how such mechanisms interact with those activated by vasoinhibins to result in specific outcomes in RA. Unraveling interactions at the level of the PRL/vasoinhibin axis will very likely help to understand the pathogenesis of RA and offer new treatment approaches to control this complex disease.

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Peptide Hormone Regulation of Angiogenesis

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Clapp C, Thebault S, Jeziorski MC, Martínez de la Escalera G. Peptide Hormone Regulation of Angiogenesis. *Physiol Rev* 89: 1177–1215, 2009; doi:10.1152/physrev.00024.2009.—It is now apparent that regulation of blood vessel growth contributes to the classical actions of hormones on development, growth, and reproduction. Endothelial cells are ideally positioned to respond to hormones, which act in concert with locally produced chemical mediators to regulate their growth, motility, function, and survival. Hormones affect angiogenesis either directly through actions on endothelial cells or indirectly by regulating proangiogenic factors like vascular endothelial growth factor. Importantly, the local microenvironment of endothelial cells can determine the outcome of hormone action on angiogenesis. Members of the growth hormone/prolactin/placental lactogen, the renin-angiotensin, and the kallikrein-kinin systems that exert stimulatory effects on angiogenesis can acquire antiangiogenic properties after undergoing proteolytic cleavage. In view of the opposing effects of hormonal fragments and precursor molecules, the regulation of the proteases responsible for specific protein cleavage represents an efficient mechanism for balancing angiogenesis. This review presents an overview of the actions on angiogenesis of the above-mentioned peptide hormonal families and addresses how specific proteolysis alters the final outcome of these actions in the context of health and disease.

I. INTRODUCTION

Blood vessels influence the metabolic effects of hormones by transporting fluid, nutrients, oxygen, and waste material. In addition, the vascular system delivers hormones from other parts of the body, allowing them to perform their local actions, which can include the production of another hormone that has to be transported to other specialized cells. Likewise, the function of blood vessels depends on hormones that regulate blood pressure, blood coagulation, and inflammation. To favor hormone delivery or blood transport into growing tissues, hormones promote angiogenesis, the proliferation of new blood vessels from preexisting vasculature. By acting systemically, hormones coordinate and integrate angiogenesis with other functions throughout the body. They also regulate blood vessel growth by controlling the production of local chemical mediators, often other hormones, but also growth factors, cytokines, enzymes, receptors, adhesion molecules, and metabolic factors, by vascular endothelial cells and other cells within the vicinity of capillaries. Notably, hormones can acquire angiogenic or antiangiogenic properties after undergoing proteolytic cleavage within the tissue microenvironment.

Proteolytic cleavage is a mechanism used frequently to generate proangiogenic and antiangiogenic protein mediators at specific sites. Proteolytic cleavage of extracellular matrix (ECM) components releases smaller proangiogenic fragments from larger proteins, as well as sequestered proangiogenic growth factors and cytokines (380). Similarly, antiangiogenic peptides are generated via proteolysis of components of the ECM and the coagulation and fibrinolytic systems (404, 519). Although proteolytically processed antiangiogenic fragments have long been known (160, 404), little attention has been given to proteolytic cleavage as an important mechanism controlling hormone action on angiogenesis.

Here, we review the regulation of angiogenesis by representative peptide hormones that are converted to proangiogenic or antiangiogenic molecules by proteolytic cleavage. The properties of these fragments versus those of their precursors, the regulation of the protease(s) responsible for specific protein cleavage, and the selective expression of specific receptors and associated signaling pathways for each hormonal isoform are discussed within a wider context of health and disease, with the expectation that understanding the role of hormones in angiogenesis could open new therapeutic perspectives for diseases resulting from angiogenic dysregulation.

II. ANGIOGENESIS OVERVIEW

The formation of new blood vessels is essential for organogenesis and successful embryonic and fetal development (139). In the adult organism, the proliferation of blood vessels is key for the growth and function of female reproductive organs, such as the ovary and endometrium during the menstrual cycle and the placenta and mammary gland during pregnancy (219). However, in most adult tissues, physiological angiogenesis is highly restricted, and capillary growth occurs only rarely and in association with repair processes such as wound and fracture healing. Disruption of the mechanisms controlling physiological angiogenesis has a major impact on health, as it underlies the pathogenesis of a growing list of diseases characterized by the overproliferation of blood vessels, including cancer, psoriasis, arthritis, retinopathies, obesity, asthma, and atherosclerosis. In addition, insufficient angiogenesis and abnormal vessel regression can lead to heart and brain ischemia, neurodegeneration, hypertension, osteoporosis, respiratory distress, preeclampsia, endometriosis, postpartum cardiomyopathy, and ovarian hyperstimulation syndrome (74, 75).

Hypoxia is an important stimulus for the physiological and pathological growth of blood vessels (449). It connects vascular oxygen supply to metabolic demand. Cells are normally oxygenated by diffused oxygen, but when tissues grow beyond the limit of oxygen diffusion, hypoxia triggers vessel growth by signaling through hypoxia-inducible transcription factors that upregulate and downregulate the expression of proangiogenic and antiangiogenic factors, respectively (449).

In the embryo, blood vessels originate from endothelial cell progenitors that migrate into avascular areas and form a primitive vessel network. This de novo formation of blood vessels, termed vasculogenesis, is followed by sprouting, branching, and stabilization in the process known as angiogenesis that utilizes existing vasculature to generate new vessels. Even though both vasculogenesis and angiogenesis have long been known to initiate a functional circulatory system during embryogenesis (267), that vasculogenesis also operates in the adult has been clarified only recently. In adults, endothelium-derived circulating cells and stem cell precursors of endothelial cells contribute to vessel growth in both physiological and pathological conditions (139, 451).

Vascular endothelial growth factor (VEGF) and angiopoietin-1 and -2 are probably the most important factors promoting angiogenesis, but the regulation of this process is complex and involves an extensive interplay between cells, multiple soluble factors, and ECM components (Fig. 1), which has been the focus of detailed reviews (5, 74, 267, 303). The following is a brief summary of the angiogenesis process illustrating some of the relevant molecular interactions.

Angiogenesis is stimulated by hypoxia, which upregulates the expression of several genes involved in different steps of angiogenesis, including VEGF, angiopoietin-2, and nitric oxide synthases (NOS) (449). Vessels dilate in response to nitric oxide (NO), and VEGF disrupts endothelial cell contacts causing vasopermeability (26). Endothelial cells are then free to migrate out of the basement membrane and through the softened perivascular space using leaked plasma proteins as a provisional matrix. However, only a subset of endothelial cells is selected for sprouting and respond to proangiogenic signals by mechanisms involving Notch receptors and their Delta-like 4 ligand (217). The directional migration of endothelial cells is primarily driven by VEGF, angiopoietin-1, angiopoietin-2, and basic fibroblast growth factor (bFGF). Other contributing cytokines include platelet-derived growth factor (PDGF), epidermal growth factor (EGF), transforming growth factor-β (TGF-β), and the guidance molecules ephrins, semaphorins, and netrins (5, 303). Proteases released by endothelial cells, including plasminogen activators, matrix metalloproteases (MMPs), heparinases, chymases, tryptases, and cathepsins, degrade and alter the composition of the ECM, allowing adequate support and guidance for endothelial cell migration. In addition to migration, sprout extension involves the proliferation of endothelial cells induced primarily by VEGF and angiopoietin-2 (in the presence of VEGF). Proteases facilitate vessel sprouting by releasing ECM-bound angiogenic activators (bFGF,

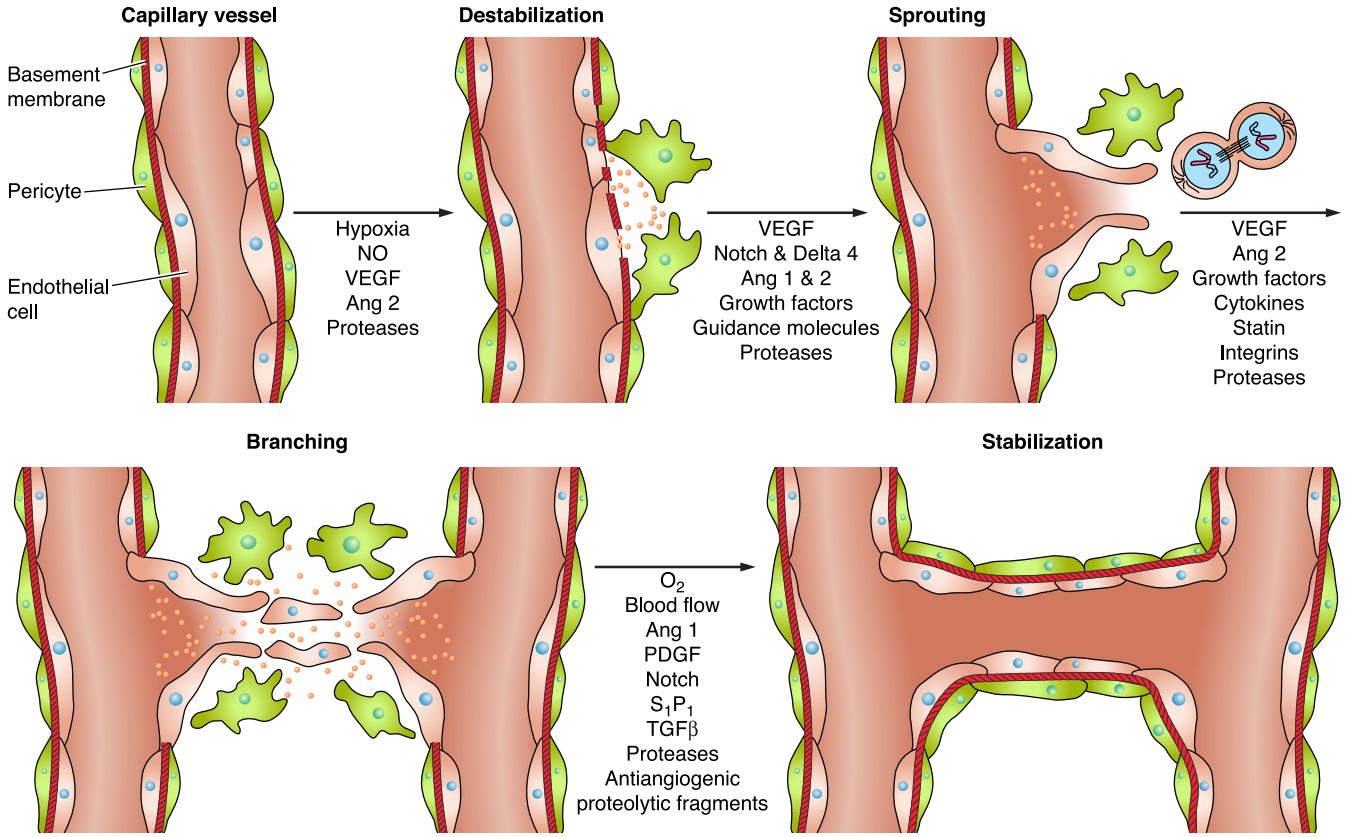

FIG. 1. The cellular steps involved in angiogenesis. Hypoxia induces the production of nitric oxide (NO) and the expression of vascular endothelial growth factor (VEGF) and angiopoietin-1 and -2 (Ang 1 and Ang 2), which interact with extracellular matrix (ECM) proteases to increase permeability of the capillary vessel wall. Destabilization then allows endothelial cells to migrate and proliferate to form tubules, aided by VEGF, angiopoietins, guidance molecules, growth factors, cytokines, and degradation of the ECM. Maturation of the newly formed vessel is accompanied by increased expression of antiangiogenic factors, many released as a result of proteolysis. PDGF, platelet-derived growth factor; S_1P_1, sphingosine-1-phosphate-1; TGFβ, transforming growth factor-β.

VEGF, TGF-β) and activating angiogenic cytokines like interleukin (IL)-1β. The degraded ECM enables integrin-mediated endothelial cell adhesion and the signaling of integrins, such as $\alpha_V\beta_3$ and $\alpha_V\beta_5$, that participate in cross-talk with VEGF and bFGF receptors to either promote or inhibit endothelial cell proliferation (491). Eventually, the sprouts join together and convert into tubes first by intracellular and then intercellular fusion of large vacuoles (273). Vascular endothelial statin, a secreted ECM-associated protein, participates in lumen formation (427). The subsequently enhanced delivery of oxygen by the onset of blood flow lowers local VEGF expression, thereby reducing endothelial cell proliferation. These events together with the recruitment of pericytes and the deposition of a subendothelial basement membrane promote vessel maturation and quiescence. These processes are regulated by angiopoietin-1 and its receptor Tie 2, PDGF and PDGF receptor β, Notch signaling (25), sphingosine-1-phosphate-1 (S_1P_1)/endothelial differentiation sphingolipid G protein-coupled receptor (16), and TGF-β (53). Proteases can also modulate and finalize the angiogenesis process by liberating ECM-bound antiangiogenic factors such as thrombospondin-1, canstatin, arresten, tumstatin, and endostatin and non-matrix-derived inhibitors of vessel formation including angiostatin, vasoinhibins, antithrombin III, prothrombin kringle-2, plasminogen kringle-5, and vasostatin (404).

III. HORMONES WITH EFFECTS ON ANGIOGENESIS ALTERED BY PROTEOLYSIS

While tremendous efforts have been devoted to the study of factors that primarily regulate angiogenesis, the contributions of broadly acting agents like hormones remain more difficult to interpret due to the quantity and diversity of their targets and actions. Hormones represent an appropriate system for regulating the angiogenesis process throughout the body. Indeed, the endocrine nature of antiangiogenic hormones enables the efficient delivery required, for example, to maintain the quiescent state of blood vessels that normally exists in adult tissues. By acting directly on endothelial cells or indirectly by recruiting other cell types to produce angiogenesis regu-

lators, proangiogenic hormones can help turn on the angiogenesis switch to promote the growth and metabolism of target tissues. Moreover, by interacting with local factors, hormones may regulate angiogenesis differentially at specific sites.

Multiple peptide hormones regulate angiogenesis by acting as either stimulatory or inhibitory factors (Table 1). Others contain, within the same molecule, the ability to exert distinct or even opposite effects on angiogenesis: proteolysis converts the original hormone to either proangiogenic or antiangiogenic peptides. The presence of opposing activities within the same hormonal precursor provides an efficient mechanism for fine-tuning angiogenesis and implies that specific proteolytic cleavage controls a shift toward a proangiogenic state or an angiostatic condition. Clearly, the selective expression of the receptors for each hormonal isoform and their related signaling pathways also play a critical role.

TABLE 1. *Hormones with angiogenic and antiangiogenic actions*

Hormone	Reference Nos.
Angiogenic	
ACTH	263
Adrenomedullin	460
Angiotensin II	236
Bradykinin	236
Calcitonin	89
Endothelin	218
Erythropoietin	23
Gastrin	212
Gonadotropins	457
Growth hormone/prolactin/placental lactogen	109
Growth hormone-releasing hormone	274
Insulin	270
IGF-I	129
Leptin	233
Neuropeptide Y	300
Oxytocin	79
Parathyroid hormone	389
Relaxin	202
Thrombopoietin	17
Thyroid-stimulating hormone	457
Vasopressin	13
Antiangiogenic	
Angiotensin-(1–7)	20
Angiotensinogen	80
Cleaved high-molecular-weight kininogen	640
des[ANG I]angiotensinogen	80
Ghrelin	29
Gonadotropin-releasing hormone	551
Natriuretic peptides	459
Somatostatin	223
Vasoinhibins	92
Pro- and antiangiogenic actions	
Adiponectin	459
Corticotropin-releasing hormone	405

The following is an overview of the actions on angiogenesis of members of the growth hormone (GH)/prolactin (PRL)/placental lactogen (PL) family, the renin-angiotensin system (RAS), and the kallikrein/kinin system (KKS), all of which are regulated by proteolysis.

A. The Growth Hormone/Prolactin/Placental Lactogen Family

GH, PRL, and PL are produced in the anterior pituitary gland as well as in the uteroplacental unit and other nonpituitary sites. They are structurally and functionally related and evolved from a common ancestral gene some 400 million years ago (107, 364, 588). They vary between 22 and 23 kDa and comprise 190–200 amino acids organized in a four-α-helix configuration stabilized by two (GH and PL) or three (PRL) disulfide bonds (Fig. 2). The relationship between their structural similarities and biological properties is unclear. Human PL and GH are remarkably similar in amino acid sequence (86% homology), size (191 amino acids), and disulfide bond number and position, whereas human PRL has 199 amino acids and 3 disulfide bonds and shares only 25% sequence identity with the other two hormones (399). Nevertheless, all three human hormones are potent agonists of the PRL receptor (204, 399), but only GH activates the GH receptor (330, 591).

GH, PRL, and PL are proangiogenic and upon proteolytic cleavage are converted to peptides with potent inhibitory effects on blood vessel growth and function, forming a family recently named vasoinhibins (Fig. 2) (92).

1. Growth hormone

GH is best known as a major regulator of linear postnatal growth, energy metabolism, muscle expansion and performance, and immune function (88, 455, 577). GH acts by stimulating insulin-like growth factor I (IGF-I) production both systemically and locally, and also by directly activating the JAK/STAT pathway in target cells (304, 309). Accumulating evidence indicates that GH helps regulate vascular growth and function. GH receptors have been detected in blood vessels from different vascular beds (296, 326, 494, 550, 603) and in cultured endothelial cells (320, 555), where GH stimulates endothelial cell proliferation (478, 529) and tube formation (67, 178). The proangiogenic effects of GH have also been demonstrated in vivo. Treatment with GH increases the number of cerebral cortical arterioles in aging rats (515), augments VEGF expression and angiogenesis in the rat myocardium after infarction (302, 468), stimulates wound angiogenesis in diabetic rats (191) and in mice (553), and may enhance vascularization by promoting mobilization

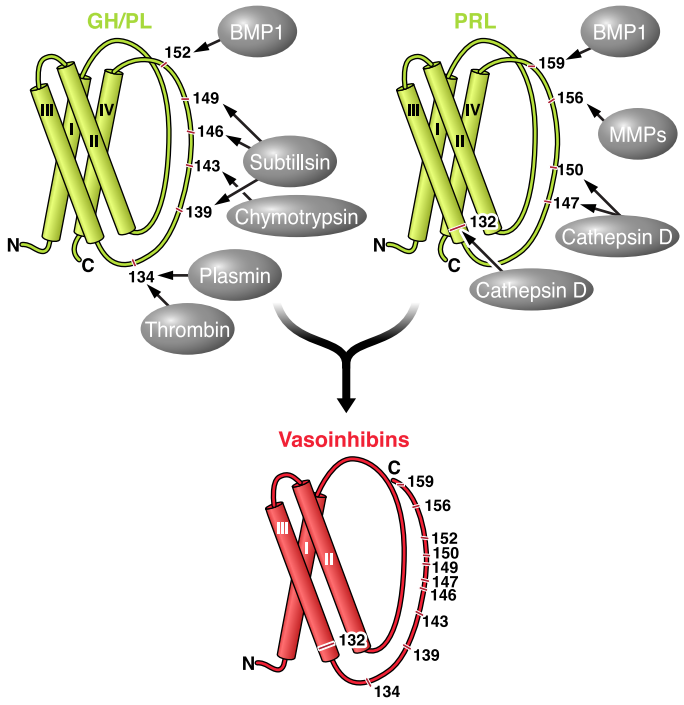

FIG. 2. The formation of antiangiogenic vasoinhibins from proangiogenic human growth hormone (GH), placental lactogen (PL), and prolactin (PRL). Each protease cleaves within the loop connecting helix III and IV (except for one cleavage site of PRL by cathepsin D), at positions indicated in amino acids, thereby generating an NH_2-terminal fragment of the indicated length in amino acids that corresponds to vasoinhibins. Vasoinhibins thus represent a heterogeneous group of antiangiogenic proteins ranging between 14 and 17 kDa. BMP1, bone morphogenetic protein 1; MMPs, matrix metalloproteases; N, amino terminal; C, carboxy terminal. Molecules are color coded in green for their angiogenic properties and in red for their anti-angiogenic effects. [Modified from Clapp et al. (92).]

of bone marrow-derived endothelial progenitor cells into the bloodstream (134, 554). Consistent with these findings, the skin of adult, GH-deficient patients shows reduced capillary density and permeability that improves after treatment with GH (418), and GH-deficient adults and children have reduced retinal vascularization (238, 239).

The retina has been considered a major target for the proangiogenic actions of the GH/IGF-I axis (for a review, see Ref. 180). Proliferative diabetic retinopathy is rare in dwarfs who are deficient for GH and IGF-I (359). Lowering circulating GH with a somatostatin analog decreases ischemia-induced retinal neovascularization in mice (510), which is also reduced by blocking GH receptor expression with antisense oligonucleotides (610) or by genetic silencing of the GH receptor in transgenic dwarf mice (510). GH action on retinal angiogenesis is mediated primarily through circulating or locally produced IGF-I (87). Administration of IGF-I to somatostatin analog-treated mice normalizes serum IGF-I levels, but not GH levels, and promotes ischemia-induced retinal neovascularization (510). Furthermore, deletion of the IGF-I receptor in vascular endothelial cells partially protects against retinal neovascularization (297), and IGF-I antagonists interfere with the actions of VEGF (511), the essential cytokine mediating proliferative retinopathies (68). Indeed, IGF-I is necessary for maximal VEGF activation of the mitogen-activated protein kinase (MAPK) and Akt pathways in the retina (511), and IGF-I induces the expression of retinal VEGF by activating the phosphatidylinositol 3-kinase (PI3-K)/Akt, hypoxia inducible factor-1 (HIF-1), NFκB, and JNK/AP-1 pathways (443).

IGF-I may also mediate the proangiogenic actions of GH at other sites, as IGF-I receptors are widely expressed in endothelial cells, and IGF-I has been shown to stimulate angiogenesis in vivo and in vitro (for a review, see Ref. 129). However, some actions of GH on endothelial cells may be independent of IGF-I. For example, GH is unable to increase the transcription of IGF-I in endothelial cells. Instead, it promotes the expression and activity of endothelial NOS (eNOS) (555), and eNOS-derived NO stimulates vasorelaxation, vasopermeability, and angiogenesis (586). In addition, systemic (320) or local infusions of GH (390) acutely increase forearm blood flow and NO release in healthy humans without significantly raising plasma IGF-I levels or muscle IGF-I mRNA expression. While these observations argue in favor of an autonomous GH action mediated by NO, IGF-I is also vasoactive due to its activation of eNOS (for a review, see Ref. 129), and more information is required to clarify this issue. The relevance of the vascular actions of GH is emphasized by clinical data showing that patients with GH deficiency have increased risk of cardiovascular death (206). Loss of GH production in humans leads to increased peripheral resistance, reduced cardiac output, decreased blood flow in response to vasodilators, and reduced systemic NO levels, whereas GH replacement restores these responses to normal (58, 73, 391). Interestingly, some vasoconstrictive effects of GH via NO inhibition have also been reported in experimental settings (370).

The complexity of GH vascular effects is further illustrated by the fact that elevated GH levels are not always associated with angiogenesis. Overexpression of GH in transgenic mice (gigantic phenotype) does not result in increased retinal neovascularization (510), acromegaly has no correlation with retinopathy (36, 167), and long-term GH replacement therapy does not appear to increase the risk of retinopathy in children or adults (239). Interestingly, in the chick embryo chorioallantoic membrane, GH is proangiogenic only during the more differentiated, nongrowing state of blood vessels (211, 529), and not in their less differentiated, proliferative phase (529). Furthermore, treatment with GH does not stimulate the proliferation of some endothelial cells in culture (67, 478). These contrasting findings imply that GH has context-dependent vascular actions influenced by other angiogenic agents, including IGF-I, NO, and VEGF,

and GH itself. GH is produced by endothelial cells, and endothelium-derived GH stimulates the proliferation, migration, survival, and capillary formation of endothelial cells in an autocrine manner (67). Therefore, the lack of action of exogenous GH may relate to endothelial cell GH receptors being already occupied by the endogenous hormone. Other important factors affecting GH actions on angiogenesis include its local proteolysis to vasoinhibins (see below) and the relative contribution of other related hormones. Human GH, the form used therapeutically and in most experimental work, has the ability to activate PRL receptors, which can also mediate proangiogenic signals.

2. Prolactin and placental lactogen

PRL was named for one of its first known functions, the initiation and maintenance of lactation, but this hormone is remarkably versatile, as it regulates various events in reproduction, osmoregulation, growth, energy metabolism, immune response, brain function, behavior (44, 59), and angiogenesis (96). In vivo studies show that treatment with PRL stimulates the proliferation of endothelial cells in the corpus luteum (195), in the testis (291), and in the myocardium (243). During pregnancy, PRL receptor deficiency interferes with the vascularization of the corpus luteum (220), and defective mammary gland development in PRL (249) and PRL receptor (419) null mice can be associated, in part, with subnormal neovascularization (96). However, several inconsistencies reflect the complexity of PRL action. Like GH, PRL is proangiogenic in the chick embryo chorioallantoic membrane assay only during the nongrowing stage of blood vessels (529), and not during their proliferative phase (94, 196, 529, 567). Also, PRL is unable to stimulate blood vessel growth in the corneal angiogenesis assay (567), and targeted disruption of the PRL gene is associated with highly vascularized pituitary tumors in aged mice (116). A major unresolved issue is whether PRL directly or indirectly stimulates endothelial cell proliferation. The PRL receptor has been detected, albeit at low levels, in some endothelial cells (360, 461, 567) but not in others (410), and the preponderance of evidence shows no mitogenic effect of PRL on cultured endothelial cells (94, 160, 410, 461, 529, 567). Nonetheless, lack of an effect could reflect the fact that endothelial cells produce and release PRL able to promote proliferation in an autocrine manner (93, 461). One study reported a direct effect of PRL on endothelial cell growth that was dependent on the expression of heme oxygenase-1 (346), an enzyme that promotes cell cycle progression and prevents apoptosis by producing bilirubin and carbon monoxide (65, 132).

On the other hand, PRL can stimulate angiogenesis indirectly by inducing the synthesis of proangiogenic factors in other cell types. PRL promotes VEGF or bFGF expression in decidual cells (517), immune cells (207, 345, 559), and mammary epithelial cells (207). The PRL-induced release of VEGF by mammary epithelial cells and the Nb2 lymphoma cell line (207) depends on the JAK2/MAPK/early growth response gene-1 pathway, whereas the activation of heme oxygenase-1 mediates PRL-stimulated VEGF transcription in macrophages (346). Evaluating PRL action is rendered more difficult because human GH and PL can also activate PRL receptors. In fact, PL signals through the PRL receptor and may be an important contributor to increased blood vessel growth during pregnancy. Like PRL, PL stimulates in vivo angiogenesis in the chick embryo chorioallantoic membrane during the nonproliferative stage of blood vessels and is unable to stimulate the in vitro proliferation of certain endothelial cells (529).

PRL can affect the function of blood vessels during injury and inflammation. PRL alters the cytoskeleton and adhesion properties of endothelial cell monolayers after mechanical injury (360) and promotes the infiltration of leukocytes (415, 547) via their integrin-mediated adhesion to vascular endothelial cells (371). Also, PRL can have stimulatory or inhibitory effects on vascular resistance, blood volume, and blood flow depending on the experimental model and condition (for a review, see Ref. 96). Importantly, the vascular actions of PRL and PL, like those of GH, can be counteracted by their proteolytic conversion to vasoinhibins.

3. Vasoinhibins

Vasoinhibins are a family of peptides derived by proteolytic cleavage from PRL, GH, and PL that inhibit blood vessel dilation, permeability, growth, and survival (reviewed in Refs. 92, 96). Cleavage by various proteases occurs near or within the large loop connecting the third and fourth α-helices in all three hormones (Fig. 2). Cathepsin D (35, 438), MMPs (MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, and MMP-13) (339), and bone morphogenic protein-1 metalloprotease (BMP-1) (196) cleave PRL to generate vasoinhibins, whereas thrombin and plasmin cleave PL (477, 529) and BMP-1, thrombin, plasmin, subtilisin, and chymotrypsin cleave GH (196, 319, 615). Since only one study has addressed the actions of vasoinhibins derived from GH and PL (529), most of the following information concerns vasoinhibins originating from PRL.

Vasoinhibins decrease angiogenesis in the chick embryo chorioallantoic membrane (94, 196, 529) and in the cornea (141); inhibit blood vessel growth and survival, vasodilation, and vasopermeability in the retina (22, 140, 192); impair growth and function of coronary vessels (243); and reduce the growth, metastasis, and neovascularization of tumors (49, 285, 398). Vasoinhibins act directly on endothelial cells to reduce the mitogenic effects of VEGF and bFGF (94, 529); inhibit bFGF- and IL-1β-

stimulated endothelial cell migration (312, 313) and tube formation (94, 313); block VEGF-induced vasopermeability (192); and prevent VEGF-, bradykinin (BK)-, and acetylcholine-stimulated vasodilation (192, 209). Vasoinhibins also promote vessel regression by stimulating apoptosis in endothelial cells (351, 537). The receptors for vasoinhibins have not been identified (97), but portions of their signaling pathways have been defined (for a review, see Ref. 96). Vasoinhibins arrest endothelial cells at G_0 and G_2 by inhibiting cyclin D1 and cyclin B1 and stimulating the cyclin-dependent kinase inhibitors p21cip1 and p27kip1 (535). This regulation may involve the inhibition of MAPK activation (117, 118) and of eNOS-dependent proliferative pathways (209, 649). Vasoinhibins also interfere with endothelial cell migration by upregulating plasminogen activator inhibitor type-1 (312) and blocking the Ras-Tiam1-Rac1-Pak1 signaling pathway (313). Vasoinhibins inhibit endothelial cell survival by activating proapoptotic proteins of the Bcl-2 family (351) and the NFκB-mediated activation of caspases (537). Finally, vasoinhibins block vasodilation and vasopermeability by interfering with the Ca^{2+}-dependent activation of eNOS (209) and by activating protein phosphatase 2A, which dephosphorylates and inactivates eNOS (192).

The extensive influence of vasoinhibins on vascular endothelial cells includes proinflammatory actions. Vasoinhibins act on endothelial cells to promote the expression of cell adhesion molecules (ICAM-1, VCAM-1, E-selectin) and of chemokines from the CXC and the CC families (536). Consequently, they stimulate the NFκB-mediated adhesion of leukocytes to endothelial cells and the infiltration of leukocytes into tumors (536). Also, vasoinhibins act as proinflammatory mediators on other cell types, like pulmonary fibroblasts, where they promote the NFκB-mediated expression of inducible NOS (iNOS) with potency comparable to the combination of IL-1β, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ (110, 340).

Vasoinhibins are emerging as natural inhibitors of the angiogenic process. They have been identified in the retina (22) and cartilage (339), where angiogenesis is highly restricted, and blocking the expression and action of vasoinhibins by siRNA targeting PRL or neutralization with antibodies results in stimulation of retinal angiogenesis and vasodilation (22). In addition, interfering with the formation of vasoinhibins by pharmacologically blocking pituitary PRL secretion prevents postpartum cardiomyopathy in mice (243). In chondrocytes, vasoinhibins appear to be generated mainly by MMPs (339), whereas other proteases predominate at different sites. Genetic deletion of two genes encoding BMP-1-like metalloproteases prevents the generation of GH- and PRL-derived vasoinhibins in mouse fibroblasts (196). Recently, cathepsin D has been shown to generate vasoinhibins within PRL secretory granules in the anterior pituitary, suggesting that vasoinhibins are released during the process of exocytosis (M. Cruz-Soto and C. Clapp, unpublished observations). Notably, vasoinhibins were not detected in the anterior pituitary glands of cathepsin D-null mice, arguing in favor of cathepsin D being the physiologically relevant protease at this site (Cruz-Soto and Clapp, unpublished observations). Understanding the mechanisms regulating the generation of pituitary vasoinhibins could help clarify conflicting data, such as the presence of highly vascularized pituitary tumors in PRL-null mice (116). Indeed, because the pituitary gland may be an important site for the cleavage of PRL into vasoinhibins (92), it is possible that the absence of vasoinhibins, rather than the lack of PRL, accounts for the upregulation of angiogenesis in pituitary tumors.

Vasoinhibins are not the only members of the PRL/GH/PL family that inhibit angiogenesis. S179D PRL, a molecular mimic of naturally occurring phosphorylated PRL, was recently shown to inhibit angiogenesis by interfering with endothelial cell migration, proliferation, survival, and growth factor signaling (567, 568). Phosphorylation of PRL at Ser-179 alters the charge of the molecule, and it will be useful to examine the structural properties of phosphorylated PRL for common features with vasoinhibins.

B. The Renin-Angiotensin System

The RAS encompasses various peptide hormones that act systemically and locally to control blood pressure and body fluid homeostasis. Over the last two decades, RAS has been a key target for the development of drugs effective for the treatment of cardiovascular diseases, including hypertension, renal diseases, cardiac hypertrophy, congestive heart failure, and ischemic heart disease (235, 349, 602).

RAS members result from stepwise enzymatic processing (Fig. 3) that starts with the cleavage of circulating angiotensinogen (AGT) by the aspartyl protease renin, forming the inactive decapeptide angiotensin I (ANG I) and the COOH-terminal protein des[ANG I]AGT. Subsequently, angiotensin-converting enzyme (ACE) cleaves ANG I to generate the main effector of RAS, the octapeptide angiotensin II (ANG II). Other endopeptidases, like neprilysin, prolylcarboxypeptidase (PrCP), and angiotensin converting enzyme-related carboxypeptidase (ACE2), cleave ANG I and ANG II to generate the heptapeptide ANG-(1–7) (311), while ACE2 also converts ANG I into the nonapeptide ANG-(1–9). Systemic ANG II is primarily produced within the pulmonary circulation, and its formation is limited by the availability of circulating renin released by the juxtaglomerular cells of the kidney. All components of RAS are present in many tissues, where ANG I and ANG II can also be produced by enzymes other than renin and ACE, including tonin, cathepsin G, chymostatin-

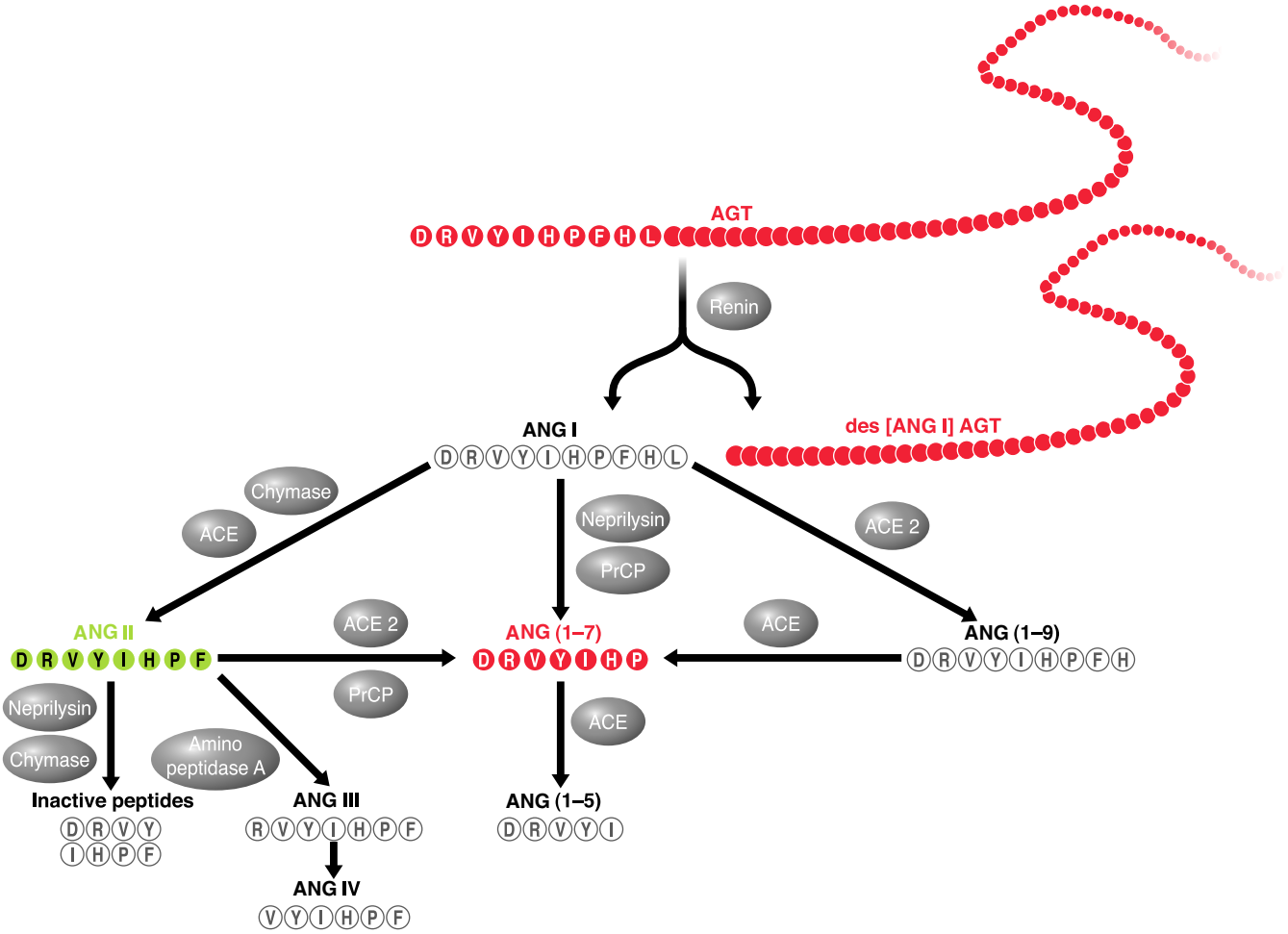

FIG. 3. Hormones of the renin-angiotensin system with effects on angiogenesis. Cleavage of angiotensinogen (AGT) by renin yields inactive angiotensin I (ANG I), a short NH_2-terminal decapeptide, and des[ANG I]AGT, which shares antiangiogenic properties with AGT. ANG I can be further processed to angiogenic angiotensin II (ANG II) and antiangiogenic angiotensin-(1–7) [ANG-(1–7)], each of which can be cleaved to yield peptides with no described effects on angiogenesis. The single-letter amino acid composition of each smaller peptide is indicated. ACE, angiotensin-converting enzyme; ACE 2, angiotensin-converting-enzyme-related carboxypeptidase; PrCP, prolylcarboxypeptidase; ANG III, angiotensin III; ANG IV, angiotensin IV; ANG-(1–5), angiotensin (1–5); ANG-(1–9), angiotensin-(1–9). Molecules are color coded in green for their angiogenic properties and in red for their antiangiogenic effects.

sensitive ANG II-generating enzyme, chymase, and tissue plasminogen activator (Fig. 3) (311). Of importance, ACE can cleave ANG-(1–9) to ANG-(1–7), and ANG-(1–7) to ANG-(1–5), while neprilysin, chymase, and aminopeptidase A inactivate ANG II.

Increasing evidence indicates that members of RAS exert both positive and negative effects on angiogenesis. Because the main role of RAS is to maintain body fluid homeostasis in response to a drop in perfusion pressure, RAS hormones likely participate in controlling neovascularization during vasoconstriction-associated ischemia. Intricate mechanisms govern their contrasting actions on angiogenesis and involve the activation of different receptor subtypes with opposite outcomes depending on specific tissue and disease conditions as well as the proteolytic conversion of larger precursor molecules, which may or may not have angiogenic actions, into smaller angiogenic or antiangiogenic peptides.

1. Angiotensin II

The most fully characterized component of the RAS system, ANG II, is normally proangiogenic when administered in vivo or in vitro (for reviews, see Refs. 236, 276). ANG II stimulates vessel proliferation in the chick embryo chorioallantoic membrane (308), the rabbit cornea (158), and the Matrigel model in mice (544). The two G protein-coupled receptors for ANG II, AT1 and AT2, have been identified in endothelial cells (126), and AT1 is the main mediator of the proangiogenic actions of ANG II. Inhibitors of AT1 block the proangiogenic effect of ANG II in the mouse Matrigel model (544), the rat ischemic hindlimb (545), and the electrically stimulated rat skeletal

muscle (18). Likewise, inhibitors of AT1, but not of AT2, prevent the stimulatory effects of ANG II on the proliferation and tube formation of cultured endothelial cells (421, 526) and bone marrow-derived endothelial progenitor cells (257). Also, AT1 antagonists inhibit the ANG II-induced upregulation of VEGF, VEGFR2, angiopoietin-2, and Tie2 in endothelial cells (90, 421) and in vascular smooth muscle cells (462). However, in kidney, both AT1 and AT2 inhibitors are effective (465). ANG II upregulates other proangiogenic mediators, including NO (416, 544), bFGF (431, 518), PDGF (106, 386), IGF-I and its receptor (63, 227, 581), EGF (186), hepatocyte growth factor (HGF) (153), and TGF-β (198, 271, 286, 414). ANG II can also directly signal endothelial cell proliferation via c-Src-mediated increase of NADP oxidase activation (563), phosphorylation of ERK1/2 (429), p38 MAP kinase (571), and JNK (488), as well as reduced phosphorylation of Src homology 2-containing inositol phosphatase 2 (SHP-2) (482).

On the other hand, ANG II is antiangiogenic under certain conditions, as inhibition of endogenous ANG II production or action by treatment with ACE inhibitors or AT1 and AT2 blockers, respectively, stimulates angiogenesis in vivo (236, 593). ANG II may inhibit angiogenesis by activating the AT2 receptor. AT2 expression is augmented in the ischemic limb of wild-type mice, and vessel density increases in the ischemic limb of AT2-knockout mice (505). Furthermore, ANG II-induced neovascularization in the cremasteric muscle is enhanced by AT2 blockers, although it is inhibited by AT1 antagonists (384). However, the contribution of each receptor subtype to the antiangiogenic effect of ANG II appears to depend on the chosen angiogenesis model. In the alginate tumor model, AT2-null mice show impaired angiogenesis, and treatment with AT1 inhibitors promotes angiogenesis (593). Interestingly, evidence for the antiangiogenic effects of ANG II via AT2 has also been provided in cultured endothelial cells (47), where AT2 receptor stimulation inhibits VEGF-induced endothelial cell migration and tube formation.

Although much needs to be learned about the mechanisms controlling the dual effects on angiogenesis of AT1 and AT2, it is generally accepted that the two receptors essentially mediate contrasting effects of ANG II (for reviews, see Refs. 124, 276). AT1 receptors are ubiquitously expressed and responsible for most of the well-known actions of ANG II, including vasoconstriction, aldosterone and vasopressin release, renal sodium and water reabsorption, sympathetic activation, augmented cardiac contractility, smooth muscle cell proliferation, vascular and cardiac hypertrophy, inflammation, and oxidative stress. In contrast, AT2 receptors are restricted to organs like the brain, kidney, adrenals, uterus, ovary, and the cardiovascular system, where their activation leads to vasodilation, lower blood pressure, reduced cardiac and vascular hypertrophy, anti-inflammation, and suppressed growth, tissue repair, and apoptosis. Each receptor subtype activates multiple signaling pathways (reviewed in Refs. 153, 276), and the opposing influence of the two receptor subtypes is supported by signaling experiments in vitro. For example, AT1 receptors upregulate the expression of VEGF and angiopoietin-2 in microvascular endothelial cells by stimulating the release of heparin-binding EGF followed by the transactivation of the EGF receptor, whereas AT2 attenuates these actions by blocking EGF receptor phosphorylation (186). Also, AT1 acts in endothelial cells through the PI3-K/Akt pathway to upregulate survivin, suppress caspase-3 activity (412), and induce focal adhesion kinase/paxillin phosphorylation (372), which lead to endothelial cell survival and migration, respectively. In contrast, AT2 blocks VEGF-induced phosphorylation of Akt, causing the inhibition of eNOS activation, endothelial cell migration, and tube formation (47). ANG II regulation of endothelium-derived NO is complicated, as both positive and negative effects of AT1 and AT2 have been reported (42, 416, 645). AT2 signals vasodilation directly by promoting eNOS activity and endothelial NO release (416), but also indirectly by stimulating the production of BK, which in turn stimulates the eNOS/NO/cGMP pathway in the endothelium (564). These AT2 actions counterbalance the vasoconstriction elicited by AT1-induced inhibition of eNOS (416), G protein-mediated activation of phospholipase C (PLC), and inositol trisphosphate-induced mobilization of intracellular Ca^{2+} in vascular smooth muscle cells (124). Other contrasting mechanisms are the AT1 activation of tyrosine kinases that phosphorylate and activate the Ras/Raf/MAPK cascade, resulting in cellular growth and survival, and the AT2 activation of MAPK phosphatase-1 that inactivates ERK1 and ERK2, leading to Bcl-2 dephosphorylation and Bax upregulation (246).

Further complexity in the regulation of angiogenesis by RAS hormones is illustrated by the fact that AGT, des[ANG I]AGT, and ANG-(1–7) are antiangiogenic.

2. Angiotensinogen and des[ANG I]angiotensinogen

The human AGT protein contains 452 amino acids; the first 10 correspond to ANG I and the remainder to des[ANG I]AGT (Fig. 3). Circulating AGT is synthesized in the liver, but AGT is also produced in tissues such as the brain, large arteries, kidney, heart, and adipose tissue, where it is hydrolyzed by extravascular renin or other proteases to ANG I or directly to ANG II (Fig. 3) (111). AGT shares structural homology with the serine protease inhibitor (serpin) family of proteins, and the fact that some serpin proteins regulate angiogenesis [angiogenin (190), pigment epithelium-derived factor (PEDF) (123), maspin (30), and cleaved antithrombin (406)] led to the discovery of the antiangiogenic properties of AGT and des[ANG I]AGT. Both proteins inhibit in vivo angiogene-

sis in the chick embryo chorioallantoic membrane and the in vitro proliferation, migration, and tube formation of endothelial cells (20, 80). No specific AGT or des[ANG I]AGT receptors have been detected, but both proteins bind to AT1 and AT2 receptors at their micromolar plasma concentrations (197). AGT does not affect the expression of angiogenic growth factors in vivo; instead, it directly suppresses proliferation of endothelial cells and induces their apoptosis (62). AGT-deficient mice exposed to cold display an abnormal vascular brain barrier phenotype (272) that may reflect blood vessels destabilized by the absence of AGT in glial cells surrounding brain capillaries (527).

Local conditions may determine whether the antiangiogenic properties of AGT and des[ANG I]AGT prevail over the proangiogenic effects of ANG II. For example, circulating AGT could prevent angiogenesis at sites lacking renin.

2. Angiotensin-(1–7)

ANG-(1–7) is present in the circulation and in tissues, where its concentration increases after any condition that raises plasma or tissue levels of ANG I (161). For example, circulating levels of ANG-(1–7) increase 25- to 50-fold during ACE inhibition (71, 294, 377) due to increased ANG I conversion to ANG-(1–7) and inhibition of ANG-(1–7) breakdown by ACE (83). Although ANG I is the primary substrate for the generation of ANG-(1–7), the latter may also be formed from ANG II (Fig. 3), and higher availability of ANG II after treatment with blockers of the AT1 receptor also elevates circulating ANG-(1–7) (71, 294, 377).

ANG-(1–7) was characterized as the first NH_2-terminal angiotensin peptide opposing the vasopressor, proliferative, and angiogenic actions of ANG II, thereby endowing RAS with greater capability for regulating tissue perfusion (161, 341, 528). ANG-(1–7) acts as a vasodilator in vascular beds of different species (161, 162, 299, 458, 486), lowers blood pressure (48), reduces the proliferation of smooth muscle vascular cells in vitro (176) and in vivo (528), is cardioprotective (483), and inhibits angiogenesis and fibrovascular tissue infiltration in the mouse sponge model (341).

Although there is some evidence that ANG-(1–7) can activate ANG II receptors (592), the G protein-coupled Mas receptor has been identified as the functional receptor for ANG-(1–7) (12). Mas is expressed in endothelial cells (119), and its targeted disruption in mice causes increased blood pressure, endothelial dysfunction, imbalance between NO and reactive oxygen species, and a major cardiovascular phenotype, all of which are consistent with lack of ANG-(1–7) signaling (625). ANG-(1–7) induces endothelium-dependent vasodilation by stimulating NO release indirectly through BK-induced eNOS activation (237, 299), but also directly by promoting Mas-mediated eNOS activation via Akt (482). In addition, ANG-(1–7) inhibits ANG II-induced phosphorylation of MAPK through prostacyclin-mediated production of cAMP and activation of cAMP-dependent kinase (542), and it prevents ANG II-induced activation of Src and its downstream targets ERK1/2 and NADPH oxidase (482). ANG-(1–7) induces as well the phosphorylation of SHP-2, which could act as a negative regulator of ANG II-induced MAPKK and Src signaling (482).

In summary, due to the dual effects of RAS hormones, local conditions affecting their synthesis and clearance rate, the production and activity of the converting proteases, and their receptor-mediated signaling pathways would determine whether an antiangiogenic or an angiogenic condition prevails. ACE inhibitors and AT1 receptor blockers, which are among the most widely prescribed drugs for blood pressure control, lower ANG II generation and action and increase renin levels, accelerating AGT cleavage into antiangiogenic des[ANG I]AGT and ANG-(1–7) (311). However, treatment with ACE inhibitors is frequently proangiogenic (for a review, see Refs. 236, 504), primarily due to blockage of ACE-induced degradation of the potent vasodilator and proangiogenic hormone BK (236, 504). RAS/BK interactions are essential for balancing blood pressure and illustrate the sophisticated local and systemic interactions influencing the final angiogenic response.

C. The Plasma Kallikrein-Kinin System

The KKS in plasma comprises the serine proteases, coagulation factor XII and plasma prekallikrein, and high-molecular-weight kininogen (HK). The plasma KKS is known as the contact system, because originally the only known mechanism for its activation was contact with artificial, negatively charged surfaces. However, it is now recognized that the contact system is activated physiologically at the cell membrane, leading to the generation of small peptides and proteins with effects on blood coagulation, inflammation, pain, natriuresis, blood pressure, vascular permeability, and angiogenesis (for reviews, see Refs. 8, 481, 487).

HK is a 120-kDa, single-chain glycoprotein composed of six domains (D1 to D6), originally identified as the protein that, upon cleavage, yields the nonapeptide BK, the main effector of the plasma KKS (266) (Fig. 4). Most plasma prekallikrein circulates bound to HK, and on the endothelial cell surface HK serves as its main binding site (381). Upon binding to the endothelial cell surface via HK, prekallikrein is converted to kallikrein by prolylcarboxypeptidase (PrCP) (495), the same serine protease that generates ANG-(1–7) and ANG II (Fig. 3). Kallikrein favors factor XII association with and activation by endo-

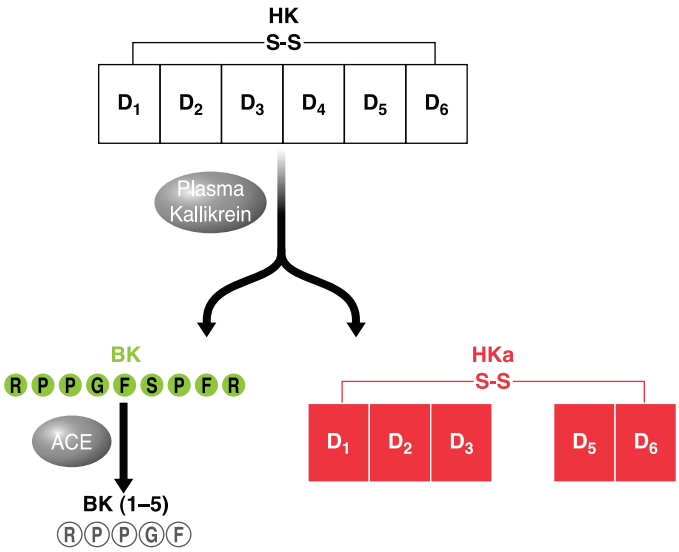

FIG. 4. Angiogenesis-related components of the kallikrein-kinin system. Domain 4 (D4) of high-molecular-weight kininogen (HK) can be cleaved by plasma kallikrein to produce the angiogenic nonapeptide bradykinin (BK) and antiangiogenic cleaved high-molecular-weight kininogen (HKa). BK can then be processed by angiotensin-converting enzyme (ACE) to yield nonangiogenic BK-(1–5). The single-letter amino acid composition of the BK peptides is indicated. S-S stands for disulfide bonds. Molecules are color coded in green for their angiogenic properties and in red for their antiangiogenic effects.

thelial cells, and activated factor XII (factor XIIa) feeds back to cleave prekallikrein to kallikrein, accelerating its formation (for a review, see Ref. 487). Kallikrein cleaves D4 in HK to release BK and cleaved HK (HKa), a two-chain structure (D1-D3 and D5-D6) linked through a single disulfide bond (Fig. 4). HK is also converted to BK and HKa by factor XIIa, albeit to a lesser extent than by plasma kallikrein (102).

Various peptidases, including ACE, metabolize BK to generate smaller peptides that have no known effect on angiogenesis (70). Pharmacological inhibitors of ACE, both by reducing ANG II levels and by blocking the degradation of BK, help control cardiovascular diseases, including hypertension and myocardial infarction (for reviews, see Refs. 236, 604). In fact, hypertension and cardiac failure have been functionally linked to impaired angiogenesis (236, 284, 604), and the plasma KKS is another hormonal system in which a protein generates both proangiogenic (BK) and antiangiogenic (HKa) fragments after undergoing proteolytic cleavage (Fig. 4).

1. Bradykinin

Discovered half a century ago, BK has multiple biological activities (reviewed in Ref. 348) and is involved in pathological states including hypertension (278) and inflammation (481). Besides being a well-known vasodilator and vasopermeability factor, BK has clear proangiogenic effects (236). It stimulates the proliferation, tube formation, and survival of cultured endothelial cells (366, 376) and promotes angiogenesis in the rabbit cornea (426), the chick embryo chorioallantoic membrane (104), the nude mouse xenograft assay (514), the rat subcutaneous-sponge model (234, 251), and the mouse ischemic hindlimb (146, 147). Moreover, angiogenesis is suppressed in HK-deficient rats and is restored by treatment with a BK analog (234).

BK stimulates angiogenesis by activating two G protein-coupled receptor subtypes, B1 and B2 (see Refs. 8, 356). Both receptors are present in endothelial cells (614), but while the B2 receptor is constitutively expressed, the B1 receptor is upregulated following tissue damage, ischemia, and inflammation (see review in Ref. 8). Actually, activation of B1 receptors may be seen as a mechanism to magnify BK actions, since this receptor subtype is normally expressed in the same cell types as the B2 receptor, uses similar signaling pathways but is less vulnerable to desensitization, and mediates the activity of other kinin metabolites (8). Inhibitors of receptors B1 (234, 321) and B2 (234, 262, 489) reduce in vivo angiogenesis and inhibit BK-induced endothelial cell proliferation (366, 376) and tube formation (366) in vitro. Notably, BK also stimulates endothelial cell growth and permeability by increasing B2 receptor-mediated VEGF expression in fibroblasts (255, 262) and smooth muscle cells (289), whereas the B1 receptor contributes to the proangiogenic action of BK by increasing bFGF synthesis in endothelial cells (137, 376, 426).

The mechanisms mediating the vascular effects of BK are not entirely understood, but it is clear that eNOS-derived NO is a major effector of BK-induced vasodilation (51, 209), vasopermeability (439), and angiogenesis (321, 426, 556). Although both B1 (426) and B2 receptors (366) signal to promote eNOS activity, B2 receptors likely predominate in mediating this action. BK stimulation of B2 receptors on endothelial cells activates PLC and phospholipase A_2, which in turn trigger the intracellular mobilization of Ca^{2+} and the activation of eNOS via calmodulin binding. In addition, BK stimulates the activation of eNOS by promoting PI3-K/Akt-induced eNOS phosphorylation at Ser-617, Ser-635, and Ser-1179 and by stimulating calcineurin-mediated eNOS dephosphorylation at Thr-497, modifications that serve to increase the Ca^{2+}-calmodulin sensitivity of the enzyme (for a review, see Ref. 579). BK also induces the phosphorylation/activation of VEGFR2, which can promote eNOS activation (366, 556).

Of importance, BK is rapidly inactivated in the intravascular compartment (half-life ~15 to 30 s; Ref. 378), and it disappears completely after a single passage through the pulmonary circulation. Its degradation is ensured by three different kininases: ACE, aminopeptidase P, and carboxypeptidase N, which cleave at positions 7–8, 1–2, and 8–9 of BK, respectively. The stable metabolic end

product of BK produced by ACE is a pentapeptide, known as BK-(1–5) (Fig. 4), that inhibits thrombin-induced platelet aggregation (385) but has no known effect on angiogenesis. BK inactivation and ANG I to ANG II conversion in the lungs are caused by the same ACE enzyme (397), and because ACE has a higher affinity for BK than for ANG I (269), the interplay between RAS and KKS must be considered to fully understand the role of both systems in angiogenesis.

Blockage of ACE stimulates angiogenesis in the heart of stroke-prone, spontaneously hypertensive rats (205) and in the rat limb muscle (69), promotes ischemia-induced angiogenesis in the rabbit hindlimb (155), and induces pseudocapillary formation and endothelial cell growth in vitro (137). The effect of BK after ACE inhibition is illustrated by the fact that neovascularization is greater in ischemic wild-type mice treated with ACE inhibitors than in the corresponding B2-receptor-null animals (504). The proangiogenic effect of ACE inhibition in the ischemic hindlimb is suppressed in B2-receptor-deficient mice (143). Importantly, the RAS and KKS interaction can also regulate angiogenesis via ANG II-dependent activation of B2 receptors (1, 383, 512, 564, 607), which leads to proangiogenic and vasodilator effects. The impact of ACE activity on RAS and BK generation and how it relates to angiogenesis regulation has been recently reviewed (236).

Predicting the outcome of KKS hormone actions on angiogenesis is complicated by the concomitant production of proangiogenic BK and antiangiogenic HKa.

2. Cleaved high-molecular-weight kininogen

HKa encompasses one heavy chain composed of D1 to D3 and a light chain corresponding to D5 and D6 linked by a disulfide bond (Fig. 4). The conversion of HK to HKa involves important conformational changes that expose a major region of D5 (599), which mediates the acquired ability of HKa to inhibit angiogenesis. HKa and recombinant D5 (also named kininostatin, Ref. 103) inhibit proliferation, migration, and survival of cultured endothelial cells (103, 226, 277, 640). Inhibition of endothelial cell proliferation is Zn^{2+} dependent and interferes with the mitogenic effects of bFGF, VEGF, HGF, and PDGF (640) by blocking the transition from G_1 to S phase of the cell cycle (226). HKa also inhibits bFGF-induced angiogenesis in the Matrigel plug assay in mice and the cornea of rats (640), and both HKa and recombinant D5 inhibit the proangiogenic effects of bFGF and VEGF in the chick embryo chorioallantoic membrane (103, 640). The antiangiogenic properties of HKa may be mediated by its interaction with urokinase receptors through the D5 region, an interaction that would compete with the urokinase plasminogen activator receptor (uPAR) for the binding of ECM proteins (vitronectin) (72). An alternative mechanism is the binding of HKa to the cytoskeletal protein tropomyosin (641), which is involved in mediating the antiangiogenic properties of endostatin, an antiangiogenic fragment of collagen XVIII (338). The proposed signaling pathways underlying the antiangiogenic effects of HKa through its D5 region include the disruption of focal adhesions via vitronectin-uPAR-$\alpha v\beta 3$-caveolin-Src-FAK-paxillin (102) or by vitronectin-uPAR-PI3K/Akt-paxillin (277), the reduction of cyclin D1 expression (226), the activation of Cdc2 kinase/cyclin A (597), and the generation of reactive oxygen species dependent on ECM components (531).

In summary, specific proteolytic cleavage of HK yields both proangiogenic (BK) and antiangiogenic (HKa) moieties. Because BK is hydrolyzed very rapidly and thus is active only close to its site of formation, BK may function as an initiator of angiogenesis, particularly in inflammatory and ischemic conditions (356), when the induction of B1 receptors can amplify its action. In contrast, HKa has a long in vivo half-life (9 h) and may counteract the angiogenic response to BK with time, tipping the balance towards antiangiogenesis. Of note, the outcome of the plasma KKS effect on angiogenesis depends not only on a repertoire of endothelial cell membrane proteins (i.e., HK-binding proteins, bound proteases, and receptors) but also on ECM proteins. HKa only induces death of proliferating endothelial cells on permissive surfaces like gelatin, fibronectin, vitronectin, and laminin (640), and its effect on vessel regression depends on the type of collagen (531). Furthermore, compensatory actions of RAS peptides may be integrated in the regulation of angiogenesis by the KKS, since the same enzyme (ACE) upregulates and downregulates the accumulation of proangiogenic ANG II and BK, respectively.

IV. HORMONAL REGULATION OF PHYSIOLOGICAL ANGIOGENESIS

Angiogenesis is normally absent in most adult organs except in female reproductive organs where intense vascular growth and regression occur physiologically, contributing to their growth, function, and involution. Members of the GH/PRL/PL family, the RAS, and the KKS regulate the physiology of reproductive organs, and some of their actions involve the control of blood vessel growth and regression.

A. Female Reproductive Organs

The endocrine system orchestrates a series of cyclical events in the female reproductive organs, allowing the ovum to mature and eventually become fertilized and the resulting new individual to be nurtured during the intrauterine and neonatal periods. Gonadotropin hormones

from the pituitary gland drive ovarian function by stimulating estrogen and progesterone production, follicle growth, ovulation, and corpus luteum development. After fertilization, the maintenance of the corpus luteum is dependent on chorionic gonadotropin and PRL, permitting the continued secretion of progesterone necessary to maintain the endometrium in a state favorable for implantation and placentation (for reviews, see Refs. 27, 525). As for the growth, differentiation, and secretory activity of the mammary gland during pregnancy and lactation, the relative contribution of PRL, GH, and PL varies among species (44), but activation of the PRL receptor is crucial (396).

In addition to these hormonal inputs, evidence indicates that GH, directly or via stimulation of IGF-I production, regulates follicular growth, sexual maturation, and luteal function (28, 636) and that ANG II produced in response to gonadotropins can stimulate follicular maturation, steroidogenesis, ovulation, and corpus luteum growth and regression (see review, Ref. 632). Also, BK has been implicated in ovulation (546, 633), and both the RAS (400) and the KKS (99) participate in regulating implantation and placentation.

Reproductive events are also determined by cyclical changes in blood vessel growth and regression within the various reproductive organs, driven primarily by VEGF and then by its blockage (for reviews, see Refs. 173, 174, 202). The transient interruption of VEGF signaling by agents specifically designed to inhibit VEGF or block its receptors suppresses follicular development, ovulation (620, 623), corpus luteum formation, progesterone release (622), and postmenstrual endometrial regeneration (156). Homozygous and heterozygous knockouts of the VEGF gene exhibit major defects in placental blood vessels (76, 159), and interference with placental VEGF compromises normal angiogenesis and leads to a poorly perfused fetoplacental unit (355). In addition, inactivation of VEGF severely limits the development and function of the mammary gland (472). The fact that female reproductive organs are under the control of hormones able to affect angiogenesis, either directly or via the expression and action of VEGF or other proangiogenic signals, strongly argues in favor of angiogenesis regulation as an essential hormonal function. We refer to recent reviews on the action of gonadotropins and steroid hormones that are considered to be important regulators of angiogenesis in reproductive organs (11, 440, 457) and keep our focus on certain proteolysis-derived peptide hormones.

1. Ovary

The information regarding ovarian angiogenesis has been extensively reviewed (172, 173, 175, 543), and accumulating evidence shows that ovarian VEGF is under hormonal control. VEGF increases in granulosa and theca cells of follicles as they become dependent on gonadotropin stimulation (501), and inhibition of gonadotropin release using a gonadotropin releasing hormone (GnRH) antagonist inhibits VEGF expression and angiogenesis in ovulatory follicles (549). Other peptide hormones stimulate follicle VEGF expression and angiogenesis. For example, IGF-I and IGF-II promote VEGF expression in granulosa cells (350), and the PRL present in follicular fluid stimulates endothelial cell proliferation (78). Of interest, a functional angiotensin system exists in the endothelial cells of the early corpus luteum (292), and ANG II induces VEGF synthesis in endothelial cells (90), promotes the expression of bFGF in bovine luteal cells (524), and may contribute to LH-induced corpus luteum angiogenesis. Furthermore, LH upregulates ovarian ANG II production (see review, Ref. 632), and ANG II antagonists can block LH-induced expression of bFGF in luteal cells (524). In addition, bFGF and VEGF upregulate luteal ANG II secretion, which supports the mechanism promoting progesterone secretion in the early corpus luteum (292).

As the corpus luteum ages in a nonfertile cycle, VEGF levels decline (422), and the angiopoietin-2/angiopoietin-1 ratio rises (203), leading to the endothelial cell apoptosis and vascular breakdown characteristic of luteolysis. In contrast, in a fertile cycle, the corpus luteum is rescued by chorionic gonadotropin, which promotes endothelial cell survival by upregulating VEGF, angiopoietin-2, and Tie-2 (621). Another important hormone regulating luteal function is PRL. In rodents, the proestrus surge of circulating PRL in a nonfertile cycle induces luteolysis, but after pseudopregnancy or pregnancy, the increase in systemic PRL promotes the survival and function of the corpus luteum (194). The opposing actions of PRL are perplexing and may involve several mechanisms (525), including both proangiogenic and antiangiogenic effects. Treatment with PRL induces endothelial cell proliferation in the corpus luteum of cycling rats, whereas reducing circulating PRL levels with bromocriptine (195) or deleting the PRL receptor gene (220) interferes with corpus luteum angiogenesis. In addition, the levels of PRL-derived vasoinhibins may rise at the end of a nonfertile cycle, because PRL increases MMP-2 activity during PRL-induced luteal regression (150), and MMP-2 generates vasoinhibins from PRL (339). Moreover, at the onset of luteal regression, corpus luteum-derived endothelial and granulosa cells produce and release higher levels of the vasoinhibin-generating protease cathepsin D (152).

2. Uterine endometrium and placenta

Angiogenesis is required for the cyclic processes of endometrial growth, breakdown, and repair during the menstrual cycle, and it provides a richly vascularized tissue receptive for implantation and placentation (reviewed in Refs. 84, 202, 265, 560, 619).

Uterine angiogenesis is regulated by multiple hormones, among which estrogen and progesterone predominate in regulating endometrial growth and differentiation. There is good evidence that estrogen drives VEGF production and angiogenesis during the proliferative phase of the cycle, whereas the presence and the absence of progesterone have been implicated in endometrial angiogenesis during the secretory and postmenstrual phases of the cycle, respectively (see reviews in Refs. 11, 202, 265). Other hormones involved in endometrial angiogenesis include chorionic gonadotropin (457), adrenomedullin (401), relaxin (208), and two subjects of this review, PRL (264) and ANG II (646).

Systemic PRL derived from the anterior pituitary gland increases during proestrus and early pregnancy in rodents, whereas in humans, circulating PRL remains low during the menstrual cycle and gradually increases during gestation, reaching its maximum level at term (reviewed in Ref. 44). PRL is also produced by the decidua (264, 447), a specialized endometrial stromal tissue that differentiates in the luteal phase of the menstrual cycle and throughout pregnancy. The location and temporal presence of PRL suggest its influence on endometrial angiogenesis during the secretory phase of the cycle and at the time of implantation and early placental development (264). Consistent with this, pharmacologically induced hyperprolactinemia enhances endometrial thickness (471) and uterine gland hyperplasia (281). PRL receptors are localized in the decidua, cytotrophoblasts, synciotriotrophoblasts (337), differentiated stromal, and uterine natural killer cells (224), which are important cellular sources of proangiogenic factors like VEGF, placental growth factor, bFGF, and angiopoietins (84, 130, 322), and PRL stimulates the expression of bFGF by decidual cells (517). Also, the placenta produces GH and PL, both of which promote endometrial gland proliferation (403, 516) and can activate PRL receptors.

On the other hand, cyclic changes in the activity of the RAS also occur during the reproductive cycle and are reflected in the circulation and in the expression of all of its components in the uteroplacental unit (reviewed in Ref. 400). During the luteal phase, ANG II increases in stromal cells near endometrial spiral arterioles, and ANG II receptor expression is increased in endometrial glands and blood vessels (9). Because ANG II stimulates the contraction of uterine blood vessels (444), ANG II may contribute to the vasopressor mechanism that initiates menstruation, causing hypoxia-induced regression and degradation of upper endometrial tissue in response to the withdrawal of progesterone (115). Also, a role for ANG II in endometrium regeneration is suggested by the presence of ANG II and ANG II receptors in endometrial glandular and stromal cells during the proliferative phase (9).

During pregnancy, all the components of RAS are active in the placenta, and increased ANG II levels in the maternal circulation are associated with placental angiogenesis and elevated blood flow (for a review, see Ref. 646). AT1 receptors are expressed predominantly in placental blood vessels and mediate ANG II-induced proliferation (55) and NO production by endothelial cells from fetoplacental arteries (646). In addition to mediating proangiogenic actions, ANG II-induced NO production helps attenuate the vasopressive response to ANG II (646). This effect is relevant, as vasodilation of maternal systemic circulation and the increased blood flow within the fetoplacental unit are major adaptations of mammalian pregnancy (509). The vasodilator response to ANG-(1–7) is enhanced during gestation (395), and systemic and renal ANG-(1–7) levels increase during late pregnancy (64, 361, 573). The different components of the KKS have also been identified in the cycling (98) and early pregnant uterus (15), suggesting that BK and its receptors actively participate in increased uterine blood flow, vasopermeability, and angiogenesis.

In addition to proangiogenic mechanisms, antiangiogenic events operate to regulate the regional distribution of placental neovascularization (reviewed in Ref. 254). Antiangiogenic mechanisms maintain the avascular nature of the decidual layer immediately surrounding the synciotrophoblastic mass of the invading embryo. Antiangiogenic events, such as endothelial cell apoptosis, also occur during human spiral arterial remodeling, which prevents maternal vessels from invading the embryonic compartment and fetal vessels from growing into the uterus. Notably, placental trophoblasts produce the secreted form of the VEGF receptor 1 (sVEGFR1), also known as soluble fms-like tyrosine kinase-1 (sFlt-1), which functions as an endogenous trap for VEGF (38, 280) and is a major contributor to decidual avascularity (254). Proteolytically modified hormones may participate in inhibiting placental angiogenesis as well. Cathepsin D is produced at the deciduo-placental interface (142, 379) and may generate vasoinhibins from decidual PRL. Indeed, amniotic fluid accumulates decidual PRL (46) and contains PRL-derived vasoinhibins, and cathepsin D from placental trophoblasts cleaves PRL to vasoinhibins (210). Likewise, the antiangiogenic vasodilator ANG-(1–7) and its generating enzyme, ACE2, are present in the decidua during early placentation when the ANG II/ANG-(1–7) ratio decreases at the implantation and interimplantation sites (395) and in various cell types of the placenta at term (574).

3. Mammary gland

The mammary gland undergoes dramatic changes in growth, differentiation, function, and involution during the reproductive cycle (see reviews in Refs. 240, 503). Pituitary gland hormones and ovarian steroids orchestrate these changes starting at puberty, when gonadotropins promote the ovarian release of estrogen and proges-

terone that stimulate the elongation and branching of the mammary gland ductal system. In addition, adrenocorticotropin hormone stimulates ductal growth in cycling females through glucocorticoid and GH release (see reviews in Refs. 250, 282). During pregnancy, progesterone, PRL, and PL promote the proliferation, differentiation, and maturation of the alveolar system responsible for milk secretion (240, 409), and while PRL appears essential for the initiation and maintenance of lactation in most species, milk production is controlled predominantly by GH in ruminants (44, 169). After weaning, involution returns the mammary gland to a virgin state, and both PRL and GH help regulate mammary gland survival by protecting it against epithelial cell apoptosis and ECM degradation (31, 168, 169).

The growth and function of the mammary gland depends on an increasing supply of oxygen, nutrients, and fluid and is therefore accompanied by the expansion of the mammary gland vasculature during pregnancy and enhanced vasodilation and vasopermeability during lactation (136, 352, 432), whereas this new vascular bed progressively disappears during the involution phase (432, 587). The importance of angiogenesis and specifically of VEGF in the mammary gland is illustrated by the fact that inactivation of the VEGF gene in the mammary gland epithelium impairs angiogenesis and blood vessel function during lactation and leads to reduced milk production and stunted growth of the offspring (472). The fact that hormones with effects on angiogenesis control the growth and function of the mammary gland makes it an attractive model for the study of the hormonal regulation of angiogenesis.

VEGF and VEGFR2 increase in the mammary gland during pregnancy and even more during lactation (432), when the influence of PRL appears to be greatest (44). PRL promotes the expression of VEGF in HC11 mouse mammary epithelial cells (207) and in immune cells associated with mammary gland involution (207, 344). Furthermore, both PRL (301, 520) and GH (450) are expressed in mammary gland epithelial cells and may regulate VEGF levels in an autocrine manner. Consistent with these findings, human MCF-7 mammary carcinoma cells overexpressing human GH produce higher levels of VEGF that promote angiogenesis in vivo and in vitro (67). The proangiogenic actions of these hormones may be counterbalanced by their conversion to vasoinhibins, since cathepsin D-mediated generation of vasoinhibins from PRL increases in the lactating mammary gland (91, 325), and the expression of various vasoinhibin-generating MMPs is upregulated during mammary gland development and involution (216). PRL, GH, PL, and vasoinhibins can also regulate mammary gland growth and function by controlling peripheral vascular resistance and blood flow to the gland. PRL can stimulate either vasodilation or vasoconstriction, and vasoinhibins have clear vasoconstrictive effects (see review in Ref. 96). These vasomotor actions also occur in response to human GH (572) and PL (221).

Likewise, members of the RAS and KKS can affect mammary gland function. BK regulates mammary gland blood flow (448, 637), and both BK and ANG II act as growth factors for normal mammary gland epithelial cells (214, 215). ANG II present in mammary gland is not necessarily derived from the circulation, as RAS components have been localized in normal mammary gland tissue, particularly in epithelial cells (538). Also, milk contains BK (613), and the isolated lactating bovine udder releases BK into the vascular perfusate (637). To our knowledge, the role of the RAS and KKS in normal mammary gland angiogenesis has not yet been investigated.

B. Nonreproductive Organs

With the exception of the female reproductive system, the vasculature of most healthy tissues is quiescent during adult life, reflecting the predominance of naturally occurring inhibitors able to counterbalance the effects of relatively abundant proangiogenic mediators. The study of this tight control of angiogenesis is particularly attractive in tissues like retina and cartilage, which are partially or totally devoid of blood vessels, respectively, and where damage to the mechanisms regulating angiogenesis contributes to the development of vasoproliferative retinopathies and arthritis.

1. Retina

In the normal adult retina, the vascularized compartment is confined to the inner organ, whereas the outer retina never becomes vascularized. Failure to inhibit blood vessel growth can result in reduced visual acuity and underlies retinopathy of prematurity, diabetic retinopathy, and age-related macular degeneration, the leading causes of blindness worldwide (642). Among the multiple regulators of retinal angiogenesis (122, 138) evidence suggests that vasoinhibins derived from PRL are crucial (see review in Ref. 95). PRL and vasoinhibins are present in ocular fluids and in the retina of rats (22, 464) and humans (140, 441) and may originate from systemic PRL (407) or from PRL synthesized locally, as PRL mRNA and protein are localized in cells throughout the retina (22, 464), including endothelial cells (410). Notably, the intravitreal injection of antibodies able to inactivate vasoinhibins promotes vessel growth in the retina, and the intraocular transfection of siRNA to block PRL expression stimulates retinal angiogenesis and vasodilation (22). Endogenous vasoinhibins may also participate in the control of vessel remodeling during development. Immunosequestering vasoinhibins in neonatal rats reduced the apoptosis-mediated regression of hyaloid vessels, a transiently existing intraocular sys-

tem of blood vessels that nourishes eye tissues during the embryonic period (140).

An important aspect of the retinal vasculature in mice is that its development begins during the first week after birth, making the murine retina a valuable model in which to study the mechanisms governing the whole angiogenesis process under physiological conditions (569). Vascularization originates at the optic nerve in the superficial layer of the inner retina and radiates towards the periphery, using a mesh of migrating astrocytes as a template and proangiogenic factors released by ganglion cells, astrocytes, and endothelial cells themselves as a guide (193, 569). GH may be among the proangiogenic factors promoting vascular development in the retina, since GH and the GH receptor are expressed in the ganglion cell layer and inner nuclear layer of the newborn mouse retina at the time when retinal vascularization occurs, and GH receptor-deficient mice show a reduction in the width of the retina and altered levels of proteins involved in retinal vascularization, i.e., protein kinase C inhibitor 1, cyclophilin A, and Sam68-like mammalian protein-2 (41). Furthermore, patients with defects in the GH/IGF-I axis exhibit reduced retinal vascularization (238, 239). The GH affecting retinal vascular development may also be derived from the circulation, since GH levels in the vitreous correlate with those in the systemic circulation in neonatal rats (43, 369). In addition, the RAS may promote retinal angiogenesis in the retina during development, when AGT, prorenin, ANG II, and the AT1 and AT2 receptors are localized in cells and blood vessels of the ganglion cell layer of the inner retina (485). The hypertensive transgenic m(Ren-2)27 rat model, in which renin and ANG II are elevated in various tissues including the retina (375), shows a more extensive development of the peripheral retinal vasculature and a reduction in vascular density in the immature retina after ACE inhibition (485).

Members of RAS are also expressed in the adult retina of mammals, including humans and rats (295, 584, 605), and there is evidence for the local production of the antiangiogenic ANG-(1–7) metabolite in the human retina (490). Furthermore, some components of the tissue KKS are expressed in neuronal cells of the outer nuclear layer, inner nuclear layer, and ganglion cell layer of the adult retina of humans (336) and rats (540), but the possible effects of counterbalancing RAS and KKS upon physiological angiogenesis in the retina remain undefined.

2. Cartilage

Cartilage is resistant to vascular invasion except during endochondral bone formation (120, 228) or in degenerative joint diseases such as rheumatoid arthritis (293). Avascularity helps provide the elasticity, flexibility, and strength of cartilage and results from the action of a variety of locally produced antiangiogenic factors that overcome the effect of multiple proangiogenic mediators (228, 500). Vasoinhibins are among the antiangiogenic factors produced in cartilage, since PRL is a component of synovial fluid (411), chondrocytes are enriched in the MMPs that convert PRL to vasoinhibins, and PRL mRNA, PRL, and vasoinhibins have been detected in articular chondrocytes (339). Articular chondrocytes also express PRL receptors, and PRL promotes chondrocyte survival (638), suggesting that both PRL and vasoinhibins act to maintain the functional integrity of cartilage.

During endochondral ossification, hypertrophic chondrocytes of the growth plate switch their phenotype from antiangiogenic to proangiogenic, producing factors including VEGF that attract blood vessels (120). The invading blood vessels bring progenitor mesenchymal cells that will later differentiate into osteoblasts and chondroclasts/osteoclasts to remodel the newly formed cartilage into bone (120). Endochondral ossification leads to longitudinal bone formation, a process governed by an intricate system of endocrine signals, including the GH/IGF-I axis (see review in Ref. 402). GH promotes longitudinal bone growth by IGF-I-independent and -dependent effects on growth plate chondrocyte proliferation and hypertrophy, respectively (595). It remains to be determined whether proangiogenic effects of the GH/IGF-I axis contribute to their actions on endochondral ossification.

V. HORMONAL CONTRIBUTION TO ANGIOGENESIS-DEPENDENT DISEASES

As previously noted, tight regulation of angiogenesis is required to prevent either excessive vascular growth or aberrant vessel regression, each of which can lead to various diseases. Consequently, the angiogenic and antiangiogenic actions of members of the GH/PRL/PL family, the RAS, and the KKS have been linked to both the etiology and treatment of angiogenesis-related pathologies.

A. Preeclampsia

Preeclampsia is defined as the onset of hypertension and proteinuria after 20 wk of gestation that, if left untreated, can progress to eclampsia, a state of generalized seizures that may harm or kill the mother or the unborn child. This disease affects 5% of all pregnancies and is a major cause of maternal, fetal, and neonatal morbidity and mortality (502). Although the etiology of preeclampsia remains undefined, it is clear that the disease depends on the placenta, as all symptoms disappear after its delivery.

Studies carried out during the last decade have shown that antiangiogenic factors produced by the pla-

centa are central to the pathophysiology of preeclampsia (for reviews, see Refs. 128, 199, 456). The syndrome is thought to arise at an early stage of pregnancy due to placental ischemia/hypoxia produced by defective trophoblast remodeling of the uterine spiral arteries. Hypoxia, in association with oxidative stress and other placental abnormalities (259, 456), stimulates the release of antiangiogenic molecules such as sVEGFR1 (247, 316, 355), soluble endoglin (sEng) (315, 318, 580), and possibly autoantibodies against AT1 receptors (AT1-AA) (259, 594). sVEGFR1 decreases free VEGF and placental growth factor levels in preeclampsia, leading to endothelial cell dysfunction and inhibition of vasodilation (331, 355). Eng is part of the TGF-β receptor complex, and sEng is antiangiogenic and inhibits eNOS-mediated vasodilation (580). Furthermore, the adenoviral expression of sVEGFR1 in pregnant rats induces the clinical signs of preeclampsia, i.e., hypertension, proteinuria, and glomerular endotheliosis (355), and the coexpression of both sVEGFR and sEng exacerbates endothelial cell dysfunction leading to severe preeclampsia, including the HELLP (hemolysis, elevated liver enzymes, low platelets) syndrome and restriction of fetal growth (580). In addition, AT1-AA with agonistic properties (57, 624) increases in the circulation of women with preeclampsia (594), and ANG II can stimulate sVEGFR1 expression in trophoblasts via AT1 receptors (647).

The influence of RAS on the pathophysiology of preeclampsia is indicated by the development of a preeclampsia-like syndrome in transgenic mice overexpressing placental renin and maternal AGT (541) and involves multiple mechanisms (see reviews in Refs. 259, 493). Although RAS components exist in the maternal and fetal placenta (108, 317) and at high levels in the circulation (33, 241), vascular responsiveness to ANG II is reduced during pregnancy (188). In contrast, components of RAS are lower in plasma (66, 305), and vascular responsiveness to ANG II is enhanced in patients with preeclampsia (417). Increased sensitivity to ANG II can contribute to preeclamptic characteristics, including abnormal trophoblast invasion and spiral artery remodeling, reduced uteroplacental blood flow, systemic vasoconstriction, and hypertension (259). Increased ANG II sensitivity may involve elevated AT1 receptor expression, which is reported to occur in the decidua of preeclamptic women (241), but, more importantly, heterodimers between AT1 receptors and BK B2 receptors are more abundant in blood vessels during preeclampsia (3). AT1-B2 heterodimers induce increased ANG II-mediated signaling in blood vessels (3) and are resistant to inactivation by oxidative stress (2, 3, 24). On the other hand, an altered production of the antiangiogenic, vasodilating ANG-(1–7) metabolite may also counterbalance the actions of ANG II in preeclampsia, since its levels increase and decrease in the circulation during normal pregnancy and preeclampsia, respectively (64, 578). Moreover, the increased vascular peripheral resistance in preeclampsia may reflect reduced responsiveness to BK, since BK-induced vasodilation of small peripheral arteries is enhanced in normal pregnancy and impaired in preeclampsia (288). However, the influences of ANG-(1–7) and BK in preeclampsia remain poorly understood.

A role for PRL in the pathogenesis of preeclampsia was suggested 30 years ago based on its osmoregulatory and hypertensive properties (248), and interest in this hypothesis was renewed with the discovery of the antiangiogenic and vasoconstrictive effects of vasoinhibins (428). Although the maternal, fetal, or amniotic fluid levels of PRL do not change in preeclampsia (333, 453), a recent study showed that urinary PRL increases in preeclamptic women in relation to the severity of the disease (310). The association between PRL levels and adverse outcomes in preeclampsia may reflect its conversion to vasoinhibins. Vasoinhibins are enhanced in the amniotic fluid, serum, and urine of preeclamptic women and may derive from the cathepsin D-mediated cleavage of PRL in the preeclamptic placenta (210, 310). Notably, the concentrations of PRL and vasoinhibins in the amniotic fluid follow an inverse correlation with birth weight in preeclampsia (210), indicating the association of these proteins with the clinical manifestation of the disease. Moreover, although much needs to be learned in this regard, there is compelling evidence that overproduction of vasoinhibins can lead to postpartum cardiomyopathy, another antiangiogenesis-dependent disease in reproduction similar to preeclampsia in that oxidative stress is a key factor for its etiology (243). In postpartum cardiomyopathy, cardiomyocyte-specific deletion of STAT-3 promotes oxidative stress, thereby enhancing cathepsin D-mediated generation of vasoinhibins, which in turn interfere with the coronary microvasculature growth and function required for adequate performance of the maternal heart during pregnancy and lactation (243).

B. Diabetic Retinopathy

Diabetic retinopathy is the leading cause of blindness in working-age individuals throughout the world. After 20 years, more than 60% of patients with type 2 diabetes and nearly all patients with type 1 diabetes develop retinopathy (171). The major risk factor is chronic hyperglycemia, which causes the apoptosis of pericytes and endothelial cells, the thickening of capillary basement membranes, and enhanced vasopermeability. Hyperpermeability leads to abnormal retinal hemodynamics and to the accumulation of extracellular fluid and hard exudates that impair vision when the macula is affected (290). Over time, intraretinal hemorrhages and capillary occlusion create areas of ischemia; the resulting retinal hypoxia leads to the production of local proangiogenic factors. In the more

advanced stages, the new blood vessels invade and bleed into the vitreous, producing a fibrovascular tissue that may result in tractional retinal detachment and blindness. The main therapy for diabetic retinopathy is laser photocoagulation, which limits vascular leakage in the retina and blocks the induction of proangiogenic factors by ablating retinal ischemic areas (628). Although laser photocoagulation is effective in preventing visual loss in many cases, it rarely improves visual acuity, may damage peripheral and night vision, and often does not quell progression of the disease (56). Thus new pharmacological therapies are being developed to target microvascular abnormalities, including anti-VEGF agents, ACE blockers, and GH inhibitors.

VEGF is a major factor enhancing vasopermeability and inducing angiogenesis in diabetic retinopathy (68). It is upregulated in the retina of animals with experimental retinopathies (363, 437), and its levels increase in the ocular fluids of patients with diabetic retinopathy (10, 507). Elevation of intraocular VEGF results in vasodilation, retinal hemorrhages, vascular leakage, and neovascularization in nondiabetic animals (100, 192, 368, 413, 558), and therapies based on the inhibition of VEGF expression and action have shown promising results as effective and safe options for the treatment of vasoproliferative retinopathies (149, 506, 628). Although anti-VEGF therapies still need to be evaluated in phase III clinical trials, their use has been authorized for age-related macular degeneration, and they are currently employed by many ophthalmologists to treat advanced diabetic retinopathy and diabetic macular edema (506).

Evidence for the role of RAS in the pathogenesis of diabetic retinopathy has been the subject of comprehensive reviews (187, 608, 609) and stems from observations showing that hypertension is a risk factor for the development and progression of diabetic retinopathy (287), that chronic hyperglycemia activates RAS (19), that RAS components are elevated in the plasma and ocular fluids of patients with diabetic retinopathy (143, 187, 608) and, importantly, that ACE inhibitors interfere with experimental retinopathy and reduce the onset and progression of the disease in humans (608). ANG II promotes diabetic retinopathy by stimulating systemic hypertension and retinal hyperperfusion and pressure, leading to shear stress-mediated release of VEGF from retinal vessels (532). However, the recent observation that an AT1 antagonist, but not antihypertensive therapy, ameliorates vascular pathology in the retina of certain diabetic rats implicates actions of RAS that are independent of high blood pressure (611). Indeed, ANG II directly stimulates the expression of VEGF and VEGFR2 in retinal cells (420, 643) and potentiates VEGF-induced proliferation of retinal capillary vessels in vitro (421). ACE inhibition reduces retinal VEGF expression and hyperpermeability (143, 200, 643) and restores retinal blood flow (245) in diabetic rats. ACE inhibition also prevents retinal neovascularization and decreases VEGF, VEGFR2, and angiopoietin-2 expression in murine ischemia-driven retinopathy (327, 374, 388, 484) in diabetic, transgenic rats overexpressing renin and AGT in the eye (375), and in the vitreous of patients with diabetic retinopathy (244).

In spite of the above experimental evidence, blockage of RAS has shown little or no effect on retinal blood vessel abnormalities in clinical trials. Improvement of diabetic retinopathy was reported after RAS blockage in patients with normotensive type 1 diabetes (85, 86, 306), but no beneficial effect was reported in patients with hypertensive type 1 diabetes (151), nor in most studies of patients with type 2 diabetes (609). The reasons for this are unclear and may involve interaction with other members of RAS and the KKS (127, 436). For example, activation of ACE2 can counterbalance the effects of ACE and ANG II by causing ANG II degradation and the production of ANG-(1–7) (Fig. 3). ACE2 expression is reduced in the diabetic retina (557), suggesting that the deleterious effect of ANG II may be exacerbated by local ACE2 deficiency. On the other hand, ACE catalyzes not only ANG II formation, but also the degradation of the vasodilator and proangiogenic peptide, BK. Recent evidence shows that BK increases vasopermeability in the healthy retina, that B1 receptors are upregulated in the retina of diabetic rats in response to oxidative stress, and that the blockage of both B1 and B2 receptors prevents the breakdown of the blood-retinal barrier associated with experimental diabetes (4). Furthermore, treatment with ACE inhibitors blocks oxidative stress and the induction of B1 receptors in diabetic rats (114), suggesting that these actions prevent the deleterious effects of BK accumulated in response to ACE inhibitors. Importantly, members of the KKS (prekallikrein, kallikrein, FXII, FXIIa, and HK) are present in the vitreous of individuals with advanced diabetic retinopathy and are activated in response to and mediate hemorrhage-induced retinal edema (189).

The contribution of the GH/IGF-I axis to the pathophysiology of diabetic retinopathy (180, 612) was described nearly four decades ago based on the elevated circulating GH levels found in diabetic subjects (275, 445, 497), the halted progression of diabetic retinopathy after anterior pituitary ablation (335) or radiation (496), and the reduced incidence of the disease in GH-deficient diabetic patients (14). Moreover, evidence that IGF-I reverses protection by a GH antagonist against ischemia-induced retinopathy (510), promotes VEGF-mediated retinal angiogenesis (443, 511), and induces most of the alterations seen in diabetic retinopathy (475) substantiates the causative role of the GH/IGF-I axis in the development of the disease.

Although IGF-I circulating levels increase as a function of the progression of diabetic retinopathy in type 1 diabetic women during pregnancy (307), most studies in

nonpregnant, diabetic patients have failed to show a relationship between circulating hormones and diabetic retinopathy. Various studies have demonstrated an increase (135, 268), decrease (157), or lack of change (181, 596) in circulating IGF-I in association with the presence or progression of the disease. Clinical trials with the GH antagonist pegvisomant yielded negative results (222), and the use of somatostatin analogs to block anterior pituitary GH secretion and to promote local somatostatin antiangiogenic actions on endothelial cells generated varying outcomes (see review in Refs. 213, 424). The reasons for these discrepancies are unclear and are likely influenced by small patient cohorts confounded not only by systemic factors such as glucose control, aggressive insulin treatment, and differential renal function, but also by ocular factors that modify the systemic incorporation or the local synthesis of GH, IGF-I, and IGF-I binding proteins (180, 612).

Little is known regarding the involvement of PRL in diabetic retinopathy (95). PRL may protect against diabetes, as suggested by the facts that PRL and PL promote the function, proliferation, and survival of β-cells (166, 177, 183), and circulating PRL levels are often decreased in poorly controlled diabetic patients (260) and rats (60). However, studies measuring circulating PRL in association with diabetic retinopathy have reported increased (373), decreased (253), or normal (81) levels. These contradictory observations may be due in part to the intraocular generation of vasoinhibins. Systemic PRL increases and gets processed to vasoinhibins in ocular fluids and in retrolental fibrovascular membranes of patients with retinopathy of prematurity (140), a neovascular eye disease caused by elevated oxygen used to improve the survival of premature neonates. Notably, vasoinhibins prevent retinal angiogenesis in ischemia-induced retinopathy (425) and inhibit the retinal vasopermeability associated with diabetic retinopathy (192). Nevertheless, studies addressing the endogenous levels of vasoinhibins and their proteolytic generation from PRL, GH, or PL in diabetic retinopathy are still needed.

C. Rheumatoid Arthritis

Rheumatoid arthritis is a chronic inflammatory autoimmune disorder of unknown etiology that targets the synovial membrane, cartilage, and bone and affects 1% of the world population (165). Autoimmunity followed by the articular infiltration of leukocytes and the hyperplasia of synovial cells lead to the development of an invasive, inflammatory front, or "pannus" that destroys the adjacent cartilage and bone. The hypoxic environment of the arthritic synovium and the action of a variety of cytokines, chemokines, and regulatory factors promote the expression of proangiogenic substances, including VEGF (113, 534). Subsequent angiogenesis facilitates the progression of the disease by enabling the continued accumulation of immune cells, expansion of the inflamed tissue, and swelling of the joints (32, 113). Indeed, blockage of VEGF action (6, 332, 365, 513) ameliorates experimental arthritis, and various antirheumatic drugs have antiangiogenic properties (576). However, no clinical approval has been obtained to use anti-VEGF therapies or other antiangiogenic therapies in patients with rheumatoid arthritis.

Epidemiological, experimental, and clinical data have linked GH and PRL to the pathophysiology of rheumatoid arthritis (reviewed in Refs. 347, 393). Interest in GH is raised by the observation that growth retardation is a major problem in juvenile rheumatoid arthritis (52), whereas emphasis on PRL derives from rheumatoid arthritis being three times more frequent in women than in men and the disease becoming exacerbated in the postpartum period in association with breast-feeding (229). Notably, both GH and PRL have immunoenhancing properties (357, 635) and restore experimental arthritis in hypophysectomized rats (50, 392); treatments with somatostatin and bromocriptine, which suppress pituitary secretion of GH and PRL, respectively, are beneficial in experimental (353, 354) and clinical (163, 164) arthritis. Levels of GH and PRL have been shown to increase in the serum (492, 562, 650) and synovial fluid (131, 387) of patients with rheumatoid arthritis. However, this issue is controversial (452, 473, 474), and no experimental evidence has demonstrated the proangiogenic actions of GH and PRL in the development of rheumatoid arthritis. Of note, the finding that MMPs capable of generating vasoinhibins are upregulated in the arthritic joint (339, 539) and that some of them are activated by locally produced PRL (387) raises the possibility that vasoinhibins generated in rheumatoid arthritis could help counterbalance the immunoenhancing and proangiogenic properties of the full-length hormones.

The contribution of RAS to the pathology of rheumatoid arthritis is suggested by the observations that ANG II has proinflammatory effects (476), that ACE, ANG II, and AT1 receptors are upregulated in synovial tissue from experimental arthritis (446) or from patients with rheumatoid arthritis (589, 590), and that targeting the angiotensin pathway with ACE blockers and AT1 inhibitors attenuates experimental arthritis (121, 479) and human inflammatory synovitis (446). Blockers of ACE activity may exacerbate inflammation by preventing the breakdown of BK. All components of the KKS have been detected in the synovial fluid of patients with rheumatoid arthritis (see review in Ref. 77) and are elevated in the peptidoglycan polysaccharide-induced rat model of arthritis and systemic inflammation (154). In this model, a BK inhibitor (105) or blockage of BK generation by an anti-HK monoclonal antibody (154) prevents joint swell-

ing and inflammation. BK inhibitors also suppress synovitis and joint erosion in collagen-induced arthritis in mice (182). The B2 receptor is present in the normal synovia, and the B1 receptor is upregulated in arthritic tissue (40, 489); the B2 receptor has been proposed to promote angiogenesis in the early stages of synovitis, whereas the B1 receptor mediates endothelial cell proliferation in the established disease (489).

D. Cancer

As in normal tissues, tumors depend on the blood supply for oxygen, nutrients, and waste removal so that in the absence of angiogenesis, tumor growth is limited to 1–2 mm in diameter (639). A key mechanism for tumor angiogenesis is local hypoxia resulting from the persistent and uncontrolled proliferation of tumor cells (231). Hypoxia triggers the expression of proangiogenic factors such as VEGF, placental growth factor, and bFGF that, when combined with oncogene-driven expression of proangiogenic peptides and inhibition of antiangiogenic agents, tilt the angiogenic balance in favor of new vessel growth (170, 498).

VEGF is overexpressed in 60% of all human cancers and is the target of antiangiogenic drugs currently used for cancer therapy that include a monoclonal anti-VEGF antibody adapted for human use (bevacizumab) and tyrosine kinase inhibitors selective for VEGF receptors (170). The survival benefits of anti-VEGF therapy are small, transitory, and accompanied by some toxic side effects (145, 283, 328). The benefits may be limited by the fact that the tumor microenvironment contains a variety of angiogenesis regulators besides VEGF (170). Insights into the role of hormones may help clarify the mechanisms mediating tumor angiogenesis and contribute to improving antiangiogenic therapies so that they continue to be effective when resistance to VEGF inhibition develops.

The contribution of pituitary gland hormones to mammary gland tumorigenesis was revealed more than 50 years ago when breast cancer was treated by hypophysectomy (334, 454). The regression of breast cancers resistant to anti-estrogen therapy following hypophysectomy (430) and the fact that PRL restored vulnerability to mammary tumorigenesis in hypophysectomized rats (21) pointed to PRL as the putative pituitary hormone essential for mammary tumor development. The participation of PRL in mammary cancer has been established in rodents, where hyperprolactinemia or PRL treatment increases the number of spontaneous mammary tumors and susceptibility to mammary carcinogens, while decreasing systemic PRL inhibits mammary tumor growth (see reviews, in Refs. 232, 583, 600). Furthermore, immunoneutralization of PRL inhibits the development of carcinogen-induced mammary tumors (362), transgenic mice overexpressing PRL develop mammary carcinomas (470, 601), and lack of PRL receptors reduces the size of mammary neoplastic growths (408).

PRL was initially considered irrelevant due to contrasting data provided by multiple clinical studies (reviewed in Ref. 101). Such variability was recently overcome by large, well-controlled studies showing that higher plasma PRL levels significantly increase the relative risk of developing breast cancer in premenopausal and postmenopausal women (230, 565, 566). In addition, epidemiological studies support the association of both dopamine antagonists and hyperprolactinemia with increased breast cancer risk (reviewed in Ref. 232). Of importance, however, neoplastic breast tissue synthesizes PRL, which is unaffected by dopamine, and it is recognized that PRL acts partly as an autocrine/paracrine promoter of mammary tumor growth (for review, see Refs. 45, 101, 583). Up to 98% of breast tumors express PRL receptors, and receptor levels are higher in neoplastic tissue than in adjacent normal tissue (201, 561). PRL stimulates the growth and survival of breast cancer cells (101, 433), and it promotes the expression of VEGF in mammary gland cell lines (207); thus increased angiogenesis may contribute to the stimulatory action of PRL on breast cancer. Mammary carcinomas in organ culture have diminished ability to generate vasoinhibins from PRL (34), and hypoxic conditions, mimicking the tumor microenvironment, are associated with reduced secretion of cathepsin D by a cultured pituitary adenoma cell line (112). These findings are in contrast to the reported greater ability of tumor cells to express and secrete cathepsin D and MMPs (423, 467), but imply that levels of vasoinhibins could be reduced in the microenvironment of tumors, thus creating a more favorable angiogenic condition for tumor progression. Notably, colon (49) and prostate (285) cancer cells overexpressing vasoinhibins generate smaller and less vascularized tumors in mice.

In addition to PRL, the GH/IGF-I axis has been shown in recent studies to play a key role in mammary cancer, particularly in animal models (see reviews in Refs. 434, 598, 627). GH restores vulnerability to mammary carcinogens in hypophysectomized rats (634) and in GH-deficient rats and mice (499, 533, 552). Disruption of the GH receptor gene inhibits oncogene-driven mammary tumorigenesis (644), and transgenic mice overexpressing a GH antagonist (442) and mice with a liver-specific IGF-I gene deletion (618) exhibit low incidence of chemically induced mammary tumors. Along this line, clinical studies have reported an association between height at various stages of development and breast cancer risk (225). High levels of both GH (148) and IGF-I (435) have been reported in the serum of breast cancer patients, and a correlation has been noted between elevated circulating IGF-I and increased risk of breast cancer development in

women (reviewed in Ref. 585). GH may act as an oncogene, since its expression in the normal human mammary gland increases with the acquisition of proliferative lesions, and the experimental overexpression of GH transforms a human, nontumorigenic mammary epithelial cell line into a tumorigenic, invasive phenotype (382, 434, 648).

In support of angiogenesis being among the tumorigenic effects of GH, receptors for GH are found in endothelial cells of newly formed tumor capillaries (550), and the overexpression of GH in human mammary carcinoma cells stimulates endothelial cell migration and tube formation in vivo dependent of VEGF (67). Also, autocrine GH downregulates the production of the antiangiogenic factor thrombospondin (626). The direct actions of GH in promoting tumor growth may involve cross-talk with the PRL receptor, which binds human GH. By overexpressing receptor-specific ligands it was shown that the PRL receptor, but not the GH receptor, mediates the high incidence of chemically induced mammary tumors in mice (601).

In contrast to PRL and GH, for which little is known in malignancies other than breast cancer (for reviews see Refs. 37, 45, 232, 627), substantial information relates RAS members to a wide variety of cancers. The extensive use of ACE inhibitors and AT1-receptor blockers to treat hypertension and congestive heart failure allowed the accumulation of clinical data that, nonetheless, is inconclusive (see reviews in Refs. 7, 323). While some studies showed that ACE inhibitors or AT1 receptor blockers reduce the incidence of cancer of the breast, female reproductive tract, lung (314), esophagus (508), and prostate (469), other studies have failed to establish such an association (179, 324, 358). Variability may relate to differences in population profiles, type of inhibitors, pharmacological parameters, and follow-up times. Also, the type of neoplasia appears relevant since the levels and activity of RAS members increase in concert with the progression of some cancers but not with others (7, 133, 153, 258, 329, 466). A naturally occurring deletion in the ACE gene leading to its elevated expression correlates with increased susceptibility to prostate (630) and breast cancer (575), but not to colorectal and lung cancer.

Experimental studies have provided stronger support for the role of RAS blockers as anticancer agents. ACE inhibitors and/or AT1 receptor antagonists inhibit tumor growth and angiogenesis in murine models of fibrosarcoma (582), renal carcinoma (242, 367), glioblastoma (463), hepatocellular carcinoma (631), head and neck squamous cell carcinoma (629), Lewis lung cancer (185), colorectal cancer metastasis to the liver (394), melanoma (125, 144), prostate cancer (298, 570), ovarian cancer (530), and gastric tumors (252). Blockage of RAS is frequently associated with reduced levels of VEGF (144, 298, 582), and both VEGF expression and action can be promoted by ANG II-induced activation of AT1 receptors (236). Studies comparing the growth and neovascularization of syngeneic tumors growing in wild-type and AT1 receptor null-mice have shown that AT1 receptors in host cells contribute to tumor growth and angiogenesis via VEGF synthesis (184, 256). In addition, long-term blockage of AT1 receptors leaves the AT2 receptor fully activated (606) and, because there is evidence that AT2 receptors mediate ANG II-induced inhibition of angiogenesis (47), the selective activation of only AT2 receptors may contribute to the antiangiogenic effect of AT1 blockers. However, AT2 receptors may also be proangiogenic (465), and an alternative pathway by which AT1 blockers may prevent angiogenesis is by increasing the concentration of ANG II and thus its conversion to antiangiogenic ANG-(1–7) (71, 294, 377). ACE inhibitors can also increase circulating ANG-(1–7) by enhancing ANG I levels and preventing ACE-mediated degradation of ANG-(1–7) (71, 83). On the other hand, inhibition of renin, leading to the accumulation of antiangiogenic AGT, may help inhibit tumor angiogenesis. Adenovirus-mediated gene transfer of AGT inhibits the growth, neovascularization, and metastasis of mammary carcinomas and melanomas in mice (61). Therefore, the outcome of RAS effects on tumor angiogenesis depends on the balance between its various metabolites with opposite effects on blood vessel growth. It is noteworthy that a common side effect of anti-VEGF cancer therapies is hypertension (328), and ACE inhibitors are frequently used in clinical trials of anti-VEGF therapies with no reference to whether these treatments also influence tumor angiogenesis (7).

In addition to interfering with the RAS system, ACE inhibitors can affect tumor growth and angiogenesis by inhibiting ACE-mediated BK degradation. Indeed, sarcoma neovascularization and growth is enhanced by the ACE inhibitor captopril, and these effects are attenuated by blocking the KKS with plasma kallikrein inhibitors or B2-receptor antagonists (261). BK is increased in plasma and ascitic fluid of some cancer patients (342, 343). Experimental data support the contribution of the KKS to tumor angiogenesis. In mice, BK enhances vascular permeability and VEGF expression in solid and ascitic tumors (262, 617), and immunoneutralization of HK to block its cleavage to BK inhibits the growth and neovascularization of human colon carcinoma cells (514) and murine multiple myeloma cells (480). Also, inoculation of Walker 256 carcinoma cells into kininogen-deficient rats that cannot generate BK results in smaller and less-vascularized tumors with a reduced VEGF content, characteristics that are mimicked by the treatment of carcinoma-bearing, normal rats with a B2-receptor antagonist (255). B2 receptors are highly upregulated in human and rodent cancer tissues (54, 616), and tumor angiogenesis and growth are markedly reduced in B2 receptor-knockout mice bearing sarcomas compared with their wild-type littermates (255).

B1 receptors are found only in malignant tumors (548), and evidence of their contribution to cancer comes from studies in mice showing that both B1 and B2 receptors are required for the growth of androgen-insensitive prostate cancer cells (39, 522) and that a BK antagonist able to block both B1 and B2 receptors inhibits lung cancer more potently than does a VEGF-receptor inhibitor (82).

Blockage of BK synthesis may also prevent generation of the other product of HK cleavage, the antiangiogenic protein HKa. Experimental work shows that the antiangiogenic domain 5 of HKa suppresses lung metastasis experimentally induced by a malignant melanoma cell line (279). There is limited experience with BK antagonists in the clinic (reviewed in Ref. 521) and none in relation to cancer. Both peptide and nonpeptide antagonists of BK are being developed that are more efficient and less toxic than many of the current anticancer drugs (523).

VI. CONCLUSIONS AND FUTURE DIRECTIONS

Members of the GH/PRL/PL family, the RAS, and the KKS regulate a wide spectrum of biological effects that depend on blood vessel number and function, including organ growth and involution, vascular permeability, and inflammation. In recent years it has become evident that these hormonal systems regulate angiogenesis by exerting both stimulatory and inhibitory influences, but the mechanisms of these actions are not well understood. The complexity of hormonal involvement is evidenced by the fact that regulation of angiogenesis depends on diverse ligands, their receptors, and their multiple signaling pathways. Also, the overlapping functions of members within and among these hormonal families influence the outcome of the angiogenic response.

Proteolytic cleavage is a mechanism shared by the PRL/GH/PL family, the RAS, and the KKS for the release of both positive and negative regulators of angiogenesis. Such cleavage can efficiently balance blood vessel growth and regression under physiological conditions, particularly in the female reproductive system, where members of these hormonal systems influence the growth and involution of the various organ structures. In addition, high circulating levels of the antiangiogenic hormonal derivatives help to maintain the quiescent state of blood vessels in adult life, while their downregulation in favor of proangiogenic metabolites contributes to angiogenesis-dependent diseases (Fig. 5).

Substantial evidence supports the action of PRL/GH/PL/vasoinhibins, RAS, and KKS in controlling angiogenesis in the reproductive organs and retina, although their putative effects in cartilage require further study. In addition, various experimental approaches including the use of powerful genetic models have highlighted the contribution of RAS in preeclampsia, of vasoinhibins in postpartum cardiomyopathy, of GH/IGF-I and RAS in diabetic retinopathy, and of PRL/GH, RAS, and KKS in cancer. The use of selective enzyme inhibitors and receptor blockers for RAS, dysfunctional mutations of the genes along the GH/IGF-I axis, and treatment with agents that target this axis at various levels have provided clinical data supporting the role of these hormones in the control of angiogenesis-related diseases.

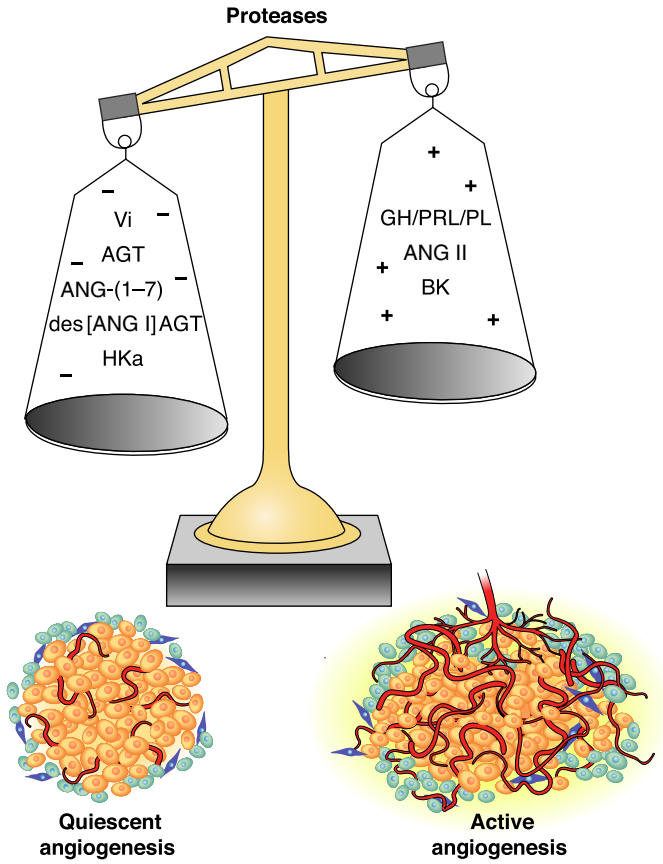

FIG. 5. Angiogenic balance as determined by protease action. The transition between quiescent and active states of angiogenesis involves the summed contribution of angiogenic and antiangiogenic factors. Within the growth hormone/prolactin/placental lactogen (GH/PRL/PL) family, the renin-angiotensin system, and the kallikrein-kinin system, the actions of proteases determine whether the net action exerted on neovascularization will be inhibitory or stimulatory. Vi, vasoinhibins; AGT, angiotensinogen; ANG II, angiotensin II; ANG-(1–7), angiotensin-(1–7); HKa, cleaved high-molecular-weight kininogen; BK, bradykinin.

Nonetheless, insufficient information concerning the regulation of angiogenesis by these hormonal families is available, few physiological and disease conditions have been examined in depth, many exceptions have been reported, and contrasting data are frequently encountered due, at least in part, to differences in animal models, tissue types, disease states, pharmacological parameters, and follow-up times. Tissue specificity is influenced by the relative proportion of stromal cells and immune cells releasing proangiogenic substances in response to hormone treatment. These effects also depend on the relative contribution of circulating versus locally produced hormones, the hor-

monal clearance rate, and the production and activity of the converting proteases. Data are surprisingly limited on the contribution of the endogenously occurring antiangiogenic moieties, i.e., vasoinhibins, AGT, des[ANG I]AGT, ANG-(1–7), and HKa, and on the presence and regulation of the converting proteases that determine their levels.

The study of the PRL/GH/PL family, the RAS, and the KKS opens numerous avenues for the pharmacological treatment of angiogenesis-related disorders. Drugs with minor side effects already available to modify the levels and effects of these hormones may be used to increase the levels of endogenous angiogenesis inhibitors or to block the action of proangiogenic mediators. So far, the complexity of these systems has prevented definitive conclusions regarding their pharmacological manipulation in human disease but has raised multiple challenges for the future. In view of the opposing effects exerted by the intact hormones and their proteolytically derived fragments, future research should focus on elucidating the mechanisms that regulate the expression and activity of the converting proteases and the selective expression of the receptors for each hormonal isoform together with their specific signaling pathways. Major research goals include the identification of functional domains affecting half-life, binding affinity, and biological potencies of the bioactive proteins to develop more specific and potent agonists and antagonists. Moreover, in-depth studies are needed to address the consequences of depleting or overproducing the various hormonal metabolites and their overlapping actions.

This review brings into focus the actions of the GH/PRL/PL family, the RAS, and the KKS on angiogenesis. Clearly, these hormonal systems are important in regulating the number and function of blood vessels, and numerous avenues of pharmacological intervention can be anticipated based on this research.

NOTE ADDED IN PROOF

During the review process of the manuscript, the work showing that cathepsin D is a primary protease for the generation of adenohypophysial vasoinhibins that was cited as unpublished observations (p. 1183) was accepted for publication (115a). Also, two seminal papers were published showing that VEGF inhibitors, while reducing primary tumor growth, promoted tumor invasiveness and metastasis (142a, 423a). These findings have raised significant concerns regarding the use of antiangiogenic therapies for cancer patients and highlight the need for a better understanding of local and systemic influences in the tumor and the blood vessel microenvironments that are altered by proangiogenic factor removal.

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From Bench to Bedside: Translating the Prolactin/Vasoinhibin Axis

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The prolactin/vasoinhibin axis defines an endocrine system, in which prolactin (PRL) and vasoinhibins regulate blood vessel growth and function, the secretion of other hormones, inflammatory and immune processes, coagulation, and behavior. The core element of the PRL/vasoinhibin axis is the generation of vasoinhibins, which consists in the proteolytic cleavage of their precursor molecule PRL. Vasoinhibins can interact with multiple different partners to mediate their effects in various tissues and anatomical compartments, indicating their pleiotropic nature. Based on accumulating knowledge about the PRL/vasoinhibin axis, two clinical trials were initiated, in which vasoinhibin levels are the target of therapeutic interventions. One trial investigates the effect of levosulpiride, a selective dopamine D2-receptor antagonist, on retinal alterations in patients with diabetic macular edema and retinopathy. The rationale of this trial is that the levosulpiride-induced hyperprolactinemia resulting in increased retinal vasoinhibins could lead to beneficiary outcomes in terms of a vasoinhibin-mediated antagonization of diabetes-induced retinal alterations. Another trial investigated the effect of bromocriptine, a dopamine D2-receptor agonist, for the treatment of peripartum cardiomyopathy. The rationale of treatment with bromocriptine is the inhibition of vasoinhibin generation by substrate depletion to prevent detrimental effects on the myocardial microvascularization. The trial demonstrated that bromocriptine treatment was associated with a high rate of left ventricular recovery and low morbidity and mortality. Therapeutic interventions into the PRL/vasoinhibin axis bear the risk of side effects in the areas of blood coagulation, blood pressure, and alterations of the mental state.

Keywords: vasoinhibins, 16K prolactin, diabetic retinopathy, diabetic macular edema, peripartum cardiomyopathy, levosulpiride, bromocriptine, dopamine D2 receptor

BACKGROUND

The prolactin/vasoinhibin axis defines an endocrine system, in which the pituitary secretion of prolactin (PRL), proteases at the central and peripheral level, and vasoinhibins at the target tissue level and in the circulation act in concert to regulate blood vessel growth and function, the secretion of other hormones, inflammatory and immune processes, coagulation, and behavior (1–5). The core element of the PRL/vasoinhibin axis is the generation process of vasoinhibins, which consists in the proteolytic cleavage of their precursor molecule PRL, the pituitary hormone essential for lactation

and colloquially referred to as the "nursing hormone." This cleavage, depending on the molecular site, removes a varying number of amino acid residues near the C-terminal end of uncleaved PRL, which corresponds to removal of at least the fourth alpha-helix of full-length PRL (6, 7). The remaining N-terminal residues assume a new, not yet resolved solution structure, and a new, unique array of endocrine, paracrine, and autocrine effects distinct from PRL (8). As the inhibition of angiogenesis was the first discovered effect, these molecules were named vasoinhibins (7, 9, 10). The generation, secretion, and regulation of vasoinhibin action integrates the hypothalamus, the pituitary, and the target tissue levels, which led to the description of the PRL/vasoinhibin axis that shares its overarching organizational principles with other endocrine axes (2). Vasoinhibins comprise a family of peptides, as multiple isoforms with variation in the number of amino acids and molecular mass, respectively, are present. The total number of vasoinhibins has yet to be determined, as well as their receptor binding sites, receptors, and complete signaling mechanisms, which are only partially known (1, 2, 4, 11, 12). Vasoinhibins act through a still-unidentified binding site in endothelial cell membranes which is distinct from the PRL-receptor (13) and can interact with multiple different partners to mediate their effects (2, 5, 14, 15). This interaction varies with the diverse effects in various tissues and anatomical compartments, indicating the pleiotropic nature of vasoinhibins (1, 2, 4). The regulation of blood vessels by PRL and vasoinhibins has been reviewed (1, 4, 12).

The accumulation of knowledge about the functions and effects of the PRL/vasoinhibin axis from basic studies has reached a critical mass which has triggered translation from bench to bedside and back, at present culminating in two clinical studies in which the PRL/vasoinhibin axis is target of therapeutic interventions to treat diabetic retinal diseases and peripartum cardiomyopathy (PPCM). It is the purpose of this review to discuss the principles behind these clinical studies, to address further areas of clinical relevance, to identify major barriers and clinical problems, and to point to solutions with which they could be overcome.

DIABETIC RETINOPATHY AND DIABETIC MACULAR EDEMA

The diabetogenic action of the pituitary has been described by Houssay and collaborators (16, 17). This seminal work was awarded by the Nobel Prize in Physiology or Medicine in 1947. A role of pituitary hormones in the etiopathology of retinal alterations emerged after observations of regression of diabetic retinopathy in a patient with Sheehan's Syndrome in 1953 (18). This has led to the use of therapies against diabetic retinopathy targeting the pituitary gland by stalk section or surgical ablation, a path which, despite beneficial retinal effects, was fortunately soon abandoned (19, 20). The beneficial retinal effects after stalk section, pituitary ablation, or Sheehan's Syndrome were, for the most part, attributed to declining levels of growth hormone and IGF-1, but circulating PRL levels were also subject of investigations addressing the etiopathology of diabetic retinopathy (21, 22). However, the results of these studies were inconsistent and did not provide sufficient mechanistic insight to delineate the actions of PRL in the diseased retina. The discovery of vasoinhibins, however, provided a new mechanistic framework and led to the reassessment of the role of PRL in the retina and its diseases. This reassessment was primarily based on the knowledge of the effects of vasoinhibins on blood vessel growth, permeability, and dilation, which correspond well with major pathological features seen in diabetic retinopathy and diabetic macular edema, for example, vascular leakage, retinal edema, intraretinal and vitreal hemorrhages, and retinal neovascularizations. How the PRL/vasoinhibin axis performs control over blood vessel growth and function at the molecular level has been the subject of two reviews, and should, therefore, not be discussed here further, but there are underlying key elements of the PRL/vasoinhibin axis at the integrative and systemic levels that are helpful for understanding ongoing clinical trials (1, 4). One of these clinical trials investigates the effect of levosulpiride on retinal alterations in patients with diabetic retinopathy and diabetic macular edema (ClinicalTrials.gov Identifier: NCT03161652). Levosulpiride, an atypical neuroleptic agent, is a benzamide derivate and a selective dopamine D2-receptor antagonist, and treatment with levosulpiride is frequently associated with the development of hyperprolactinemia. The development of hyperprolactinemia with levosulpiride is due to blockage of dopamine receptors on the pituitary lactotrophs mediating inhibition of PRL-release (23). A low dose of levosulpiride is used as a prokinetic agent (24–26). Levosulpiride-induced hyperprolactinemia is usually an unintended side effect and can be accompanied by decreased libido, erectile dysfunction in men, and galactorrhea and amenorrhea in women. The clinical study on the effect of levosulpiride on retinal alterations in patients with diabetic retinopathy and diabetic macular edema, however, is an attempt to exploit positive effects of hyperprolactinemia, induced by a low dose of levosulpiride, on retinal outcomes. The principal finding that led to the development of this concept was a study in rats, in which the induction of hyperprolactinemia resulted in vasoinhibin accumulation in the retina and a reduction of vascular endothelial growth factor (VEGF)- and diabetes-induced retinal vasopermeability was demonstrated (27). The effect could not be observed in rats with genetic deletion of the PRL-receptor; also, the effects could be blocked by bromocriptine, which lowered the levels of circulating PRL and retinal vasoinhibins. Thus, the study indicated that circulating PRL can be incorporated into the eye and cleaved to vasoinhibins intraocularly, which could lead to beneficiary outcomes in terms of a vasoinhibin-mediated antagonization of VEGF- and diabetes-induced retinal vasopermeability (**Figure 1A**; **Table 1**). In consequence, it appeared that the counteraction of angiogenic factors, such as VEGF, and of excessive vasopermeability by the raising of ocular vasoinhibins, constitute direct therapeutic interventions into pathological pathways associated with the development of diabetic retinopathy and diabetic macular edema. The development of this trial is also embedded into a long history of studies portraying the eye and its structures as targets for PRL and vasoinhibins (14, 28–40). The completion of this randomized, placebo-controlled clinical trial, which is carried out in Mexico and currently in the recruiting phase, will demonstrate whether this concept can safely and effectively be translated to its clinical application.

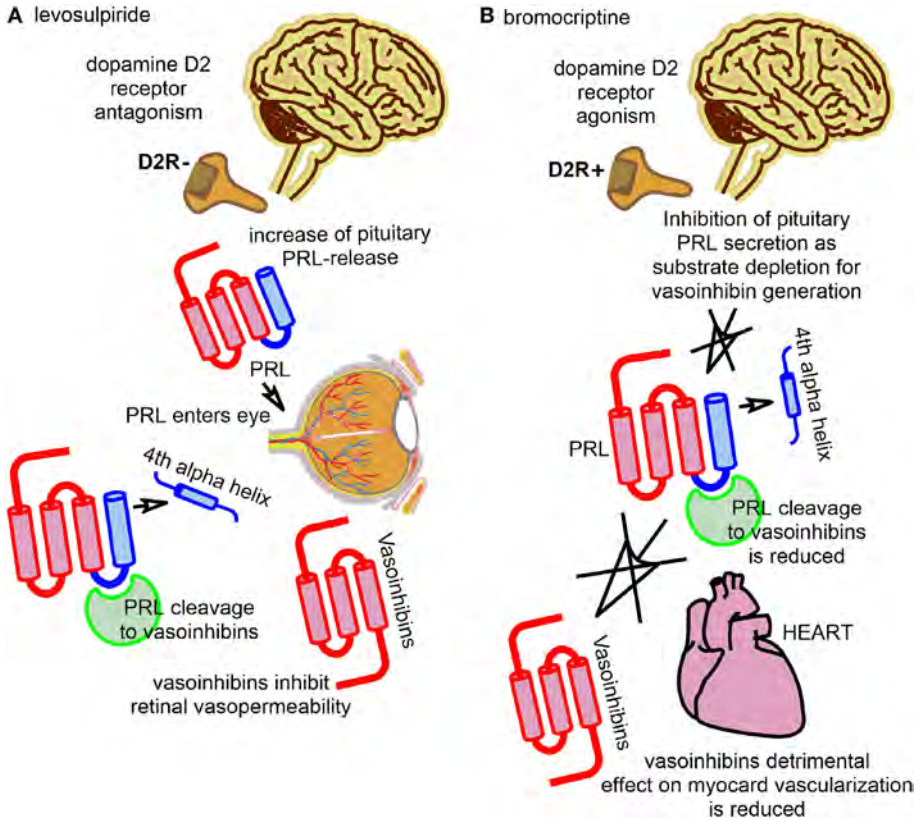

FIGURE 1 | The figure illustrates the key principles employed by two current clinical studies with interventions into the regulation of the prolactin/vasoinhibin axis axis. **(A)** One trial evaluates the effect of levosulpiride on retinal outcomes in patients with diabetic macular edema and diabetic retinopathy. Levosulpiride, a dopamine D2-receptor antagonist, is used to induce an increase of pituitary prolactin (PRL)-secretion *via* antagonization of the inhibiting effect of dopamine on PRL-secretion (disinhibition of PRL-release). PRL can enter the eye and be cleaved to vasoinhibins, with beneficial effects in retinal outcomes in terms of reducing retinal vasopermeability and their vascular endothelial growth factor-antagonism. **(B)** Another trial evaluated the effect of bromocriptine on the left ventricular function in patients with peripartum cardiomyopathy. Bromocriptine was used to inhibit pituitary PRL-secretion by dopamine D2-receptor agonism. Vasoinhibins can no longer be produced by proteolytic cleavage of PRL, and their detrimental effect of the vascularization of the myocard is reduced.

TABLE 1 | Current clinical studies with interventions into to the regulation of the prolactin/vasoinhibin axis.

Disease	Clinical pathology	Proposed pathomechanism	Therapeutic intention	Therapeutic strategy	Drug	ClinicalTrials.gov Identifier
Diabetic retinopathy and diabetic macular edema	Retinal edema	Increase in retinal vasopermeability	Inhibition of retinal vasopermeability by vasoinhibins	Increase PRL-secretion by dopamine D2-receptor antagonism	Levosulpiride	NCT03161652
Peripartum cardiomyopathy	Low left ventricular ejection fraction	Vasoinhibin-mediated damage of myocardial vascularization	Inhibition of vasoinhibin generation in the heart	Inhibition of PRL-secretion by dopamine D2-receptor agonism	Bromocriptine	NCT00998556

PERIPARTUM CARDIOMYOPATHY

A role for PRL in the etiopathology of heart failure, and PPCM in particular, was suggested in case reports in 1979 and 1984 (41, 42). However, the data remained inconclusive, particularly because a mechanism by which PRL could exert detrimental effects on the heart was not known. The discovery of cleaved PRL in 1980 in rats (43, 44) and its detection in humans 1985 (45), the identification of the anti-angiogenic effects of a 16 kDa PRL fragment in the early 1990s (9, 10), the generation of a 16 kDa PRL by cathepsin D (46), the discovery of more anti-angiogenic PRL-fragments (7, 47), and their subsequent classification as vasoinhibins (7, 48, 49), provided the framework for a study from 2007 (50), in which it was suggested that an excessive generation of vasoinhibins in the heart could impair the myocardial microvascularization and contribute to the development of PPCM. Indeed, PRL levels during the peripartum/postpartum period can be up to 20 times higher than normal, in order to facilitate lactation (51). This appears as

a precondition for a vasoinhibin-related onset of PPCM, as PRL is the immediate precursor molecule of vasoinhibins, and high substrate (PRL) availability favors the enzymatic generation of vasoinhibins. A second precondition for the excessive vasoinhibin generation in PPCM appears to be a high activity of the PRL-cleaving enzyme cathepsin D, which, in combination with the elevated PRL levels, is proposed to lead to abnormally high vasoinhibin values in the heart, detrimental effects on the vascularization of the myocardium, and subsequent development of heart insufficiency. It is reported that vasoinhibins lead to an increased level of microRNA-146a expression in endothelial cells, which exerts angiostatic effects and impairs the metabolic activity of cardiomyocytes (52). More detailed molecular descriptions of the pathways, including information on possible factors involved in the myocardial signal transduction of vasoinhibins, can be found in the original papers (50, 52) and have also been reviewed (53). Based on these insights, a new therapeutic approach for PPCM was developed, using the dopamine D2-receptor agonist bromocriptine; a drug usually applied in patients with a prolactinoma or Parkinson's disease. The principle behind this approach is the inhibition of vasoinhibin generation by substrate depletion, or the inhibition of pituitary PRL-secretion by lactotrophs, respectively (**Figure 1B**; **Table 1**). Pilot studies using bromocriptine as an add-on treatment to standard heart failure therapy reported possible beneficial effects with a normalization of left ventricular functions and dimensions (54–56). A proof-of-concept study for the evaluation of bromocriptine appeared to confirm the positive effects of bromocriptine, and a randomized, controlled multicenter clinical trial to evaluate the effect of bromocriptine in patients with PPCM, conducted in Germany, was then initiated (ClinicalTrials.gov Identifier: NCT00998556) (57, 58). The trial has recently been completed and the results demonstrated that bromocriptine treatment was associated with a high rate of left ventricular recovery and low morbidity and mortality (59).

POTENTIAL RISKS ASSOCIATED WITH THERAPEUTIC INTERVENTIONS

Therapeutic intervention of the PRL/vasoinhibin axis is likely to be associated with risks that complicate the clinical decision to commence therapy with both D2R-antagonists and agonists, or stimulating/blocking vasoinhibin generation and/or signaling by other means (60). These risks can be inferred from the known profile of biological effects of vasoinhibins, but may also include unexpected side effects and complications that can only be identified in clinical studies. Relevant issues, for example, are the effects of vasoinhibin stimulation or blockage on blood coagulation, as well as possible effects on blood pressure. Plasminogen activator inhibitor-1 was recently identified as a frequent binding partner of vasoinhibins, and this binding is responsible for the mediation of profibrinolytic effects of vasoinhibins (5). Blocking vasoinhibin production and/or signaling could, therefore, contribute to the formation or stabilization of thrombi. Of note, histological analysis of lung sections demonstrated a higher number of thrombi in control mice than in vasoinhibin treated mice (5). The clinical relevance of this observation is—at present—unclear, but it points to the importance of vigilance toward thrombotic events in patients in which inhibition of vasoinhibin generation and/or signaling is the target of intervention, for example, when inhibiting vasoinhibin generation with bromocriptine in patients with PPCM (60). Likewise, elevating PRL and/or vasoinhibin levels, as in the trial evaluating levosulpiride for the treatment of diabetic macular edema and retinopathy, could include delayed and disturbed coagulation. In mice, vasoinhibins have been demonstrated to be able to upregulate blood pressure by modulating the activity of endothelial nitric oxide synthase (eNOS) (61). Hence, blood pressure fluctuations may be due to changes in vasoinhibin levels and could appear when vasoinhibin levels are manipulated. Indeed, some of the cardiovascular side effects of bromocriptine, such as hypotension, syncope, and pleural/pericardial effusion, could be influenced by a decline of vasoinhibin levels (60).

The range of possible side effects when intervening the PRL/vasoinhibin axis also includes effects on the mental state. These effects are implied by experiments in rodents, demonstrating that the intraventricular administration of vasoinhibins leads to an increase in anxiety and depression-related behaviors (3). This scenario is further implied by an investigation showing a high prevalence of depression in women with PPCM, as the higher circulating vasoinhibin levels in these patients may enter the cerebrospinal fluid and exert neuropeptide-like effects in the central nervous system (62, 63). Lastly, the occurrence of maniac episodes after the initiation of medication with cabergoline and bromocriptine (64) may be related to central vasoinhibin levels, as a sudden decline of vasoinhibins may contribute to elevated arousal and affect (63). Of note, Ergot-derived drugs, such as bromocriptine, can induce retroperitoneal fibrosis and pleural, pericardial, and cardiac valve fibrotic reactions (65).

MAJOR BARRIERS

The major barrier not yet overcome, which delays a more thorough, more in-depth clinical evaluation of vasoinhibins is the lack of a quantitative assay for the determination of vasoinhibins in biological fluids, such as serum, plasma, cerebrospinal fluid, urine, and tissue homogenates. Some experimental techniques, such as mass spectrometry, have been evaluated, but the only more widely used methodology for detecting vasoinhibins is immunoprecipitation with anti-PRL antibodies and subsequent Western blotting (28, 50, 66). This technique has multiple limitations, including a relatively low sensitivity and a relative lack of quantitative information, and is, in most cases, not precise enough to unambiguously discriminate between vasoinhibin isoforms. The presence of multiple vasoinhibin isoforms of different molecular masses is a challenge for the development of a quantitative immunoassay, as it complicates the decision of which isoform should be targeted when monoclonal anti-vasoinhibin antibodies are manufactured. This challenge would be alleviated, if there would only be one dominating isoform being associated with a particular disease, such as PPCM or preeclampsia, and the other isoforms would not be produced, or only be present in negligible amounts. However, in contrast to *in vitro* and *in vivo* experimental studies, no clinical study has provided clear proof of the exact identity of the vasoinhibin isoform under investigation, that is their complete amino acid sequence or cleavage site within

the PRL sequence, which could then be used as the template to produce monoclonal anti-vasoinhibin antibodies. Moreover, several clinical studies reported the association of changes in vasoinhibin levels of more than one isoform at the same time, indicating that, according to disease state, more than one isoform may be involved (28, 67). These observations extend to another unmet challenge requiring attention: the site of vasoinhibin production and the controlling mechanisms determining their overall isoform composition. Vasoinhibins are generated in the pituitary gland and in multiple peripheral tissues and fluids (2, 68), but information about which of these sites is the one producing vasoinhibins measured in the circulation of patients is not available. For example, elevated serum levels of vasoinhibins in patients with PPCM might derive from PRL cleavage occurring in the heart, but may also originate from another site of vasoinhibin generation. This problem is relevant for clinical investigations, as some reports correlate the serum activity of PRL-cleaving, vasoinhibin generating enzymes with circulating vasoinhibin levels, implying that vasoinhibins are either produced in the circulation, or that the enzyme activity in the circulation corresponds with its activity at the site of vasoinhibin generation, for example, at the organ or tissue level (50, 69). Both possibilities are not supported by evidence and, thus, require clarification. Moreover, questions about the controlling mechanisms of single vasoinhibin isoforms production arise when only one cathepsin D-, or MMP-cleaved isoform, is detected (50). These enzymes use multiple cleavage sites within the PRL sequence to generate vasoinhibins of varying molecular mass, and if only a single isoform is produced, unknown controlling mechanisms must be in place suppressing the generation of the other isoforms (70). Of note, the quantitative determination of vasoinhibin levels is a missing piece in the characterization of the role of vasoinhibins in diabetic retinopathy and PPCM, but also in other diseases that have been brought into context with a dysregulation of vasoinhibins, and only if vasoinhibin levels are evaluated, their role in the aforementioned diseases can be further substantiated.

PERSPECTIVES

The present time is unique in the scientific history of PRL research, as new entities—diabetic retinal diseases and PPCM—are added to the short list of conditions in which the pituitary secretion of PRL is target of therapeutic interventions. This list had previously comprised only the condition of prolactinoma and the inhibition or PRL-release for ablactation or secondary amenorrhea. Of note, there are more clinical entities in which studies reported that a dysregulation of PRL and of the PRL/vasoinhibin axis might play a role, for example, breast and prostate cancer (71–75), preeclampsia and eclampsia (67, 76, 77), pregnancy-induced hypertension (78), pulmonary artery hypertension (79), retinopathy of prematurity (28), and rheumatoid arthritis (80). These conditions require thorough clinical investigation, including determination of PRL and vasoinhibin levels, and, ideally, additional experimental validation. In due course, in case the role of the PRL/vasoinhibin axis in these diseases is consolidated, it is possible that altering PRL and vasoinhibin levels represents a new option for therapeutic intervention. However, a better understanding of the physiological regulation of this axis and of its alterations under such diseases is required, as too many factors are still unclear. These factors comprise, as discussed, the sites and regulatory mechanisms involved in vasoinhibin generation, the relative contribution of vasoinhibins isoforms generated not only by proteolytic cleavage of PRL but also by the cleavage of related hormones, such as growth hormone and placental lactogen (47, 81). Undoubtedly, new information about the solution structure of vasoinhibins, their bioactive domains, receptors and signaling mechanisms, and the evolutionary emergence of the various isoforms (2, 8, 11) are required to advance the field in the future and to substantiate the impact of the PRL/vasoinhibin axis in human health and disease.

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JT wrote the manuscript. MR-O, RG-F, GE, CC, and TB edited and revised the manuscript. All authors approved the final version of the manuscript.

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Principles of the prolactin/vasoinhibin axis

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Triebel J, Bertsch T, Bollheimer C, Rios-Barrera D, Pearce CF, Hüfner M, Martínez de la Escalera G, Clapp C. Principles of the prolactin/vasoinhibin axis. *Am J Physiol Regul Integr Comp Physiol* 309: R1193–R1203, 2015. First published August 26, 2015; doi:10.1152/ajpregu.00256.2015.—The hormonal family of vasoinhibins, which derive from the anterior pituitary hormone prolactin, are known for their inhibiting effects on blood vessel growth, vasopermeability, and vasodilation. As pleiotropic hormones, vasoinhibins act in multiple target organs and tissues. The generation, secretion, and regulation of vasoinhibins are embedded into the organizational principle of an axis, which integrates the hypothalamus, the pituitary, and the target tissue microenvironment. This axis is designated as the prolactin/vasoinhibin axis. Disturbances of the prolactin/vasoinhibin axis are associated with the pathogenesis of retinal and cardiac diseases and with diseases occurring during pregnancy. New phylogenetical, physiological, and clinical implications are discussed.

prolactin; vasoinhibins; 16K prolactin; prolactin-fragments; prolactin/vasoinhibin axis

VASOINHIBINS are a novel family of hormones that are known for their antiangiogenic, antivasopermeability, and antivasodilation effects (16, 22–24, 31). Vasoinhibins derive from the pituitary hormone prolactin (PRL), as they are generated through the proteolytic cleavage of this hormone. PRL acts as a classical pituitary hormone, and, structurally, corresponds to a long-chain class-I helical cytokine. Like all other class-I helical cytokines, PRL folds into a bundle of four α-helices (57, 61, 94). Class-I helical cytokines signal via related receptors that share structural signatures (9, 95) and activate similar intracellular signaling pathways (93, 101). Despite their origin, vasoinhibins seem to have little in common with the typical characteristics of long chain class-I helical cytokines as they do not appear to activate similar intracellular signaling pathways and have different effects from their precursor counterpart (22, 23). Their set of vascular effects (inhibition of angiogenesis, vasodilation, and vasopermeability) is unique and entirely different from the effects of the precursor PRL molecule. It is this structural and functional distinctiveness that confers the identity to the vasoinhibin family.

Vasoinhibins, as defined by their characterization as a family, are not a single species, instead their molecular mass ranges between 11 and 18 kDa. This is due to the generation of vasoinhibins through proteolytic cleavage of PRL by several endogenous proteolytic enzymes, namely cathepsin D (8, 79), matrix metalloproteinases (69), and bone morphogenetic protein-1 (BMP-1) (45). Since these enzymes cleave full-length PRL at various sites near or within the long loop connecting the third and the fourth α-helix, proteolysis by these enzymes results in the synthesis of vasoinhibins of varying molecular masses. Vasoinhibins all share the NH_2-terminal region of full-length PRL. The COOH-terminal fragment that arises from the proteolysis of PRL does not possess vasoinhibin-like activities (62).

The roots of discovery of vasoinhibins date back to the year 1980 when PRL fragments were first detected in extracts of the rodent pituitary cells (73). Soon thereafter, PRL fragments were found in the human pituitary gland and plasma (87). It was observed that PRL fragments are not only present in the pituitary gland, but are also generated in vitro by the rat prostate gland (27, 28), mammary gland, kidney, and liver (15, 108). At this time, their function was yet unknown. However, it was already assumed that their presence is not due to coincidental, unspecific breakdown of PRL, but instead that these PRL fragments have specific physiological significance (15). It was the discovery of the inhibiting effect that these PRL fragments exert on the proliferation of bovine capillary endothelial cells and human umbilical vein endothelial cells that first demonstrated their physiological relevance (19, 39). Continued work over the ensuing years provided further insights into the function of these PRL fragments, until, in 2006,

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they were characterized as a family and received the designation "vasoinhibins" inspired by one of their principal effects, the inhibition of angiogenesis (16, 17, 69). Today, vasoinhibins are known as a new family of hormones which, in addition to their physiological role in regulating vascular function and growth (23), also seem to be involved in the pathogenesis of diabetic complications (5, 44, 80, 98, 99), cancer (63, 74), and pregnancy-associated diseases (47, 51, 54, 64, 65, 70, 78).

Generation, Secretion, and Regulation

Vasoinhibin generation in the pituitary gland occurs by proteolytic cleavage of their immediate precursor PRL by cathepsin D. Studies in rodents revealed that vasoinhibins are generated in the adenohypophysis, where, in the secretory granules of PRL, cathepsin D cleaves full-length PRL to yield vasoinhibins (34). Pituitary vasoinhibin generation is thus closely intertwined with PRL production. This is imperative, as vasoinhibins are not a product of a separate mRNA (73, 85), but are generated by a posttranslational modification, proteolytic cleavage, of PRL. However, the ratio of vasoinhibin generation to PRL synthesis is not fixed, instead it varies under physiological control. For example, the female, virgin rat features a pituitary vasoinhibin-to-PRL ratio of 0.22. During pregnancy, however, this ratio increases to 0.37 on *day* 9 and further to 0.77 on *day 12* (73). Consistent with these observations, plasma levels of vasoinhibins during the third trimester of human pregnancy are higher compared with nonpregnant states (87). The ratio of vasoinhibin to PRL synthesis is accessible to pharmacological manipulation, as demonstrated by the increase of the vasoinhibin-to-PRL ratio from 0.22 in the nontreated rat, to 0.99 after treatment with perphenazine, a dopamine D1 and D2 receptor antagonist that stimulates the production and release of PRL by the pituitary gland (73). The ratio is also increased by treatment with estrogen and reduced by thyrotropin releasing hormone (TRH) (34, 40, 73). Thus vasoinhibins possess the characteristics of effector hormones secreted from the anterior pituitary gland, similar to PRL, except that they feature posttranslational modification from the latter. These series of events, expression and posttranslational modification of PRL, and subsequent secretion by exocytosis resemble the generation of other hormones generated from a prohormone. Parallel examples are the generation of the adrenocorticotropic hormone (ACTH) and it's related peptide hormones generated in the corticotroph cells of the adenohypophysis by proteolytic cleavage of their precursor proopiomelanocortin (POMC) (105) and the thyroid hormone axis (TRH, TSH, T4/T3), where the bioactive T3 is processed from T4 by local deiodinases at the target tissue level.

It is also evident that vasoinhibin levels at the target tissue are regulated by the abundance of circulating PRL secreted by the pituitary gland. This is demonstrated by the observation that the induction of hyperprolactinaemia in rodents leads to vasoinhibin accumulation within the retina. Vice versa, pharmacological inhibition of pituitary PRL secretion with the dopamine agonist bromocriptine lowers the level of retinal vasoinhibins (5). Another situation in which vasoinhibin level at the target tissue depend on systemic PRL concentration is demonstrated in rodent studies investigating the role of vasoinhibins in peripartum cardiomyopathy (PPCM). Female mice receiving a chronic infusion of recombinant PRL had higher left ventricular myocardial vasoinhibin levels than controls (54).

The third tier at which vasoinhibin levels are regulated, next to intrapituitary regulation of the vasoinhibin-to-PRL ratio and total pituitary PRL secretion, is the expression and activity of the PRL-converting enzymes at the target tissue level. This is observed in mice with experimentally induced PPCM, where higher left ventricular vasoinhibin levels associate with increased protein concentration and activity of cathepsin D (54). This is also observed in the placenta of women with diabetes mellitus Type 1, in which, compared with controls, increased vasoinhibin generation associates with a higher expression of the PRL-cleaving enzyme BMP-1. Of note, placental mRNA PRL expression is also higher, suggesting that upregulation of local PRL synthesis can serve the purpose of providing sufficient amounts of substrate required for local vasoinhibin generation (78).

Three principles can be derived from the above observations. First, vasoinhibins are generated in the anterior pituitary gland from which they are secreted as effector hormones. Second, the central, anterior pituitary generation of vasoinhibins is under physiological control over the total anterior pituitary PRL production and activity of the PRL converting proteases. Third, the regulation of vasoinhibin concentration at the target tissue level includes the utilization of circulating and locally produced PRL and the level of activity of local PRL cleaving enzymes. Thus it appears that generation, secretion, and regulation of vasoinhibin action demonstrate the organizational principle of an axis that integrates the hypothalamus, the pituitary, and the target tissue level (Fig. 1). Indeed, this corresponds with the classical three tiers of control that subserve the regulation of anterior pituitary hormone secretion (82). Furthermore, the generation of vasoinhibins at the target tissue level represents a novel example of paracrinology, an exciting concept in endocrinology by which hormone action is being regulated at the target tissue microenvironment (56).

Molecular Species, Distribution, and Target Tissues

Cleavage of human PRL by cathepsin D results in the generation of vasoinhibins comprising residues 1-80-85 (11 kDa),1–150 (17.2 kDa), 1–147 (16.8 kDa), and 1–132 (15 kDa) (79). Cleavage of PRL by matrix metalloproteinases (MMP) results in vasoinhibins comprising residues 1–150 (17.7 kDa), 1–132 (16.8 kDa), 1–124 (14.1 kDa), and 1–111 (12.5 kDa) (69). MMP cleave PRL to generate vasoinhibins with the following relative potency: MMP-8 > MMP-13 > MMP-3 > MMP-1 > MMP-2 > MMP-9 (69). Proteolysis of PRL by BMP-1 generates a single vasoinhibin species comprising residue 1–152 (17 kDa) (45) (Fig. 2). Oxidative stress can increase the activity of cathepsin D to cleave PRL (54), whereas hypoxia decreases cathepsin D-induced vasoinhibin generation (33). A higher expression of cathepsin D and a parallel increase in vasoinhibin generation is also observed during mammary gland involution (58). The activity of MMP from chondrocytes to generate vasoinhibins seems to be higher in patients with arthritis (69), pointing toward the possibility of an induction of vasoinhibin generation by inflammatory factors. Factors associated with the activity of BMP-1 in the generation of vasoinhibins are not known.

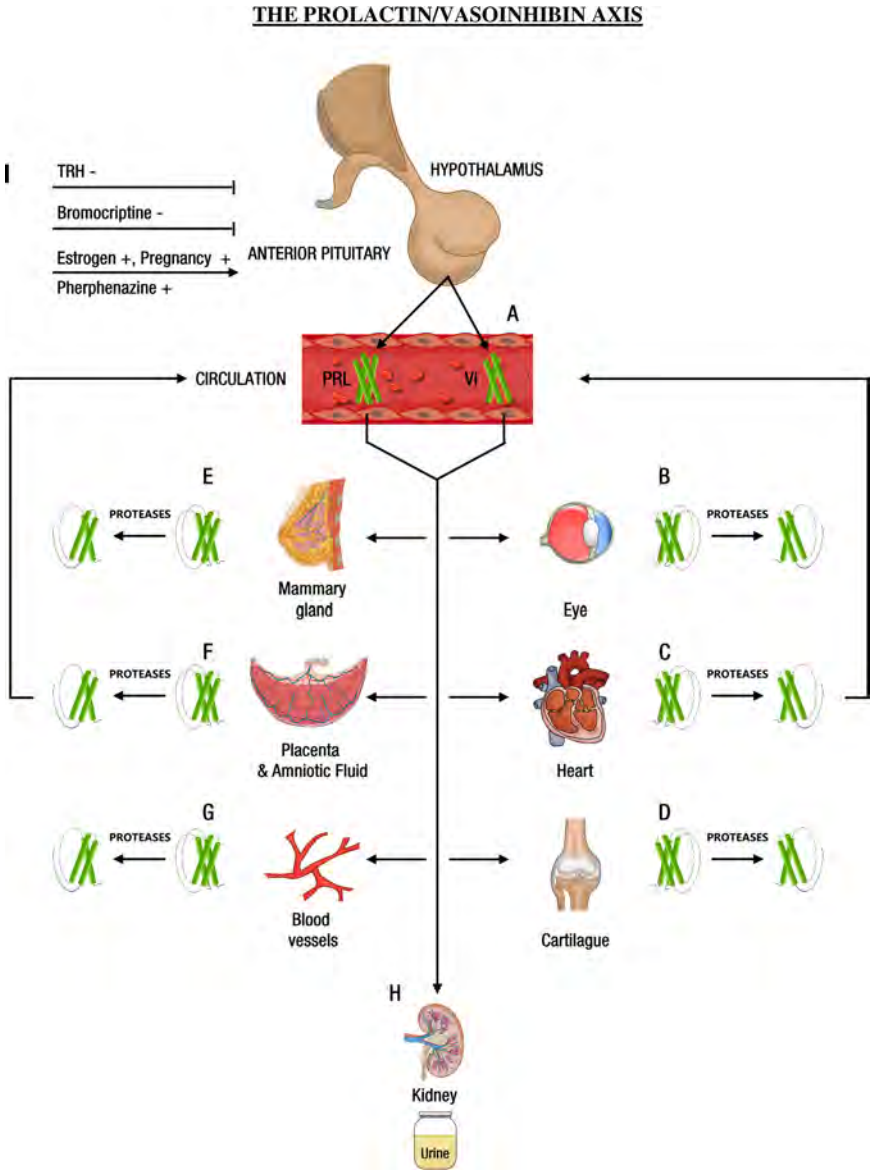

Fig. 1. Central and peripheral regulation of the prolactin/vasoinhibin axis. *A*: anterior pituitary. Prolactin (PRL) and vasoinhibins (Vi) are secreted from the anterior pituitary gland. *B*: Eye. Hyperprolactinemia leads to vasoinhibin accumulation in the retina and inhibition of pituitary PRL secretion with the dopamine agonist bromocriptine lowers retinal vasoinhibins. A dysregulation of retinal/ocular vasoinhibins is linked to vasoproliferative retinopathies. *C*: Heart. Higher circulating PRL levels lead to higher left ventricular myocardial vasoinhibin levels. Local activity of cathepsin D regulates local vasoinhibin levels. An excessive myocardial vasoinhibin synthesis is linked to peripartum cardiomyopathy. Vasoinhibins generated in the heart can enter the circulation. *D*: Cartilage. Matrix metalloproteinases in cartilage generate vasoinhibins from circulating and cartilage-produced PRL. *E*: Mammary gland. Local activity of cathepsin D regulates local vasoinhibin levels. Vasoinhibins participate in mammary gland involution. *F:* Placenta and amniotic fluid. Local activity of bone morphogenetic protein-1 (BMP-1 and cathepsin D and upregulation of placental mRNA PRL expression regulate local vasoinhibin synthesis. An excessive, dysregulated placental vasoinhibin synthesis is linked to preeclampsia, fetal growth abnormalities, and maternal diabetes mellitus. Vasoinhibins generated in the placenta can enter the circulation. *G*: Endothelium. Endothelial cells express PRL mRNA and generate vasoinhibins. The role of circulating PRL and vasoinhibins on the levels of vasoinhibins at the endothelium is unclear. *H*: Kidney. Vasoinhibins appear in the urine of women with preeclampsia, pointing toward an altered renal elimination of vasoinhibins under pathophysiological conditions. *I*: Central regulation. Thyrotropin releasing hormone (TRH) and bromocriptine inhibit the synthesis of vasoinhibins in the anterior pituitary gland. Estrogen, the state of pregnancy and the antipsychotic drug perphenazine stimulate the synthesis of vasoinhibins in the anterior pituitary.

Vasoinhibin species are detected in the rat (34, 73), mouse (86), and human (79, 87) pituitary gland, the human vascular endothelium (30), the human and rat placenta (78), the bovine corpus luteum (38), and the following rodent tissues: hypothalamus (110), neurohypophysis (25), cartilage (69), and retina (4, 5). However, the placental localization of vasoinhibins may be questioned since placental samples can be contaminated with decidua, and both human and rodent decidua produce PRL (52, 76). Of note, in vitro studies demonstrate that these and the following tissues generate vasoinhibins when exposed to full-length PRL: heart (54), ventral prostate, spleen, lung, kidney and mammary gland (8, 15, 27, 28, 58, 108) in rodents, and the mammary gland (7) and placenta (47) in humans. Vasoinhibin species are also detected in human and rat plasma/serum (37,

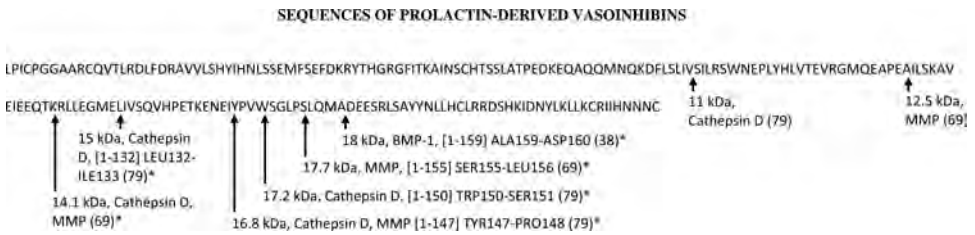

Fig. 2. The protein sequences of full-length PRL and of PRL-derived vasoinhibins are presented, including information on their molecular mass and enzyme involved in the vasoinhibin generation and cleavage sites (indicated with arrows). The number in brackets indicate the residues comprising the respective vasoinhibin molecule. Where available, the cleavage site is also indicated. *Vasoinhibins that have been tested for anti-angiogenic activity.

47, 87, 98), in human amniotic fluid (43, 47), subretinal fluid (37), and urine (47, 65). A summary of the distribution and functions of vasoinhibins in the human and the rodent organism is presented in Table 1.

The presence of vasoinhibins in the pituitary gland and plasma reflect their hormonal nature, that is, their generation and secretion by an endocrine gland into the circulation. However, vasoinhibins can also act locally at their production site, as demonstrated in rodent studies in which antiproliferative and proapoptotic actions of vasoinhibins were observed in the anterior pituitary gland (40). In case of the hypothalamic paraventricular nuclei and the supraoptic nucleus, it appears that vasoinhibins function as stimulators of vasopressin release (71) and as antagonists of vascular endothelial growth factor (VEGF), whose expression is particularly high at these sites (25, 60). An antagonism to the effects of VEGF may also operate in cartilage, an avascular tissue (69), and is observed in the rat retina, in which vasoinhibins reduce VEGF- and diabetes-induced retinal vasopermeability (5, 44). Indeed, because of the close anatomical vicinity of vascularized tissues and avascular compartments, the eye, in which vasoinhibins control ocular angiogenesis, vasodilation, and vascular permeability, is one of the best-characterized target organs for vasoinhibins and represents a key illustrative example of the actions of vasoinhibins (4, 21, 22, 44, 77, 99). However, as these functions of vasoinhibins and their clinical implications in retinal diseases have recently been reviewed (21, 22, 99), they will not be subject of this review. Rats receiving the intracerebroventricular administration of vasoinhibins show anxious and depressive behaviors (110), suggesting that vasoinhibins may be local regulators of neuronal function.

Whether the effects of vasoinhibins in the above-mentioned target tissues are due to their action on the vessel system in

Table 1. *Distribution and functions of endogenous vasoinhibins in humans, rodents, and other organisms*

Anatomical Location	Endogenous Vasoinhibins		Vasoinhibin Generation		Vasoinhibin Mass (kDa)/Protease		Established or *Assumed* Functions	Ref. No.
	Human	Rodent	Human	Rodent/other	Human	Rodent		
Central Nervous System								
Pituitary gland	yes	yes	—	yes	16	16/CD	Generation, secretion, ↓ proliferation, ↑ apoptosis	34, 73, 79, 86, 87
Neurohypophysis	—	yes	—	yes	—	14	*VEGF-antagonism*	25
Hypothalamus	—	yes	—	yes	—	14	*VEGF-antagonism* ↑ Anxiety, ↑ depression	(25, 71, 110)
						17	↑ Vasopressin release	
Retina	—	yes	—	yes	—	16	↓ Permeability, ↓ vasodilation	(4, 5)
Fibrovascular membranes	yes	—	—	—	16	—	↑ Vascular regression	(37)
Body Fluids								
Plasma/serum	yes	yes	—	—	18; 16; 14	14	Transport, ↓ *vasopermeability*, ↓ *vasodilation*	(31, 37, 47, 87, 96, 98)
Amniotic fluid	yes	—	yes	—	14/CD	—	VEGF-antagonism	(43, 47)
Subretinal fluid	yes	—	yes	—	16/NP	—	↑ Vascular regression	(37)
Urine	yes	—	—	—	18; 16; 14	—	*Elimination*	(47)
Organs/Tissues								
Placenta	yes	yes	yes	—	17	16	*Anti-angiogenesis*	(47, 70, 78)
					15	14		
Mammary gland	—	yes	—	yes	—	16, 14/CD	↑ Involution	(15, 58, 108)
Corpus luteum	—	yes	—	yes/bovine	—	16/CD	↑ *Angioregression*	(38)
						14/CD		
Endothelial cells	yes	—	yes	yes/bovine	16, 14	—	↓ Proliferation, ↓ migration	(18, 30)
Fibroblasts	—	yes	—	yes	—	17/BMP-1	Antiangiogenesis	(32, 45, 69)
						16/CD		
Heart	—	—	—	yes	—	16/CD	↓ Cardiomyocyte metabolism, anti-angiogenesis	(54)
Liver	—	—	—	yes	—	16	Unknown	(15)
Cartilage/chondrocytes	—	—	yes	yes	17,16,14/MMP	17,16,14/MMP	*Antiangiogenesis*	(69)
						14/CD		
Prostate	—	—	—	yes	—	16/CD	Unknown	(27, 28)
Testes/sperm	—	yes	—	—	—	16,17,18	Unknown	(59)

The anatomical locations with corresponding evidence in regard to the occurrence of endogenous vasoinhibins, the ability to generate vasoinhibins from prolactin (PRL), the vasoinhibin molecular species, and the protease involved are presented. Established functions relates to in vitro and/or in vivo evidence in the cited literature, whereas assumed function (in italic) relates to putative functions not supported by in vitro or in vivo data. CD. cathepsin D, MMP. matrix metalloproteinases; NP, neutral proteases. –Not reported.

these tissues or on a tissue-specific cell-type is not always clear. The vasoinhibin effect in the heart, however, in which vasoinhibins utilize the cardiomyocyte to exert their effect on the heart's vasculature, points to the possibility of a complex interplay between both, vascular and nonvascular cells. Furthermore, the proportion as to which the total amount of vasoinhibins present in a target tissue is of systemic or local origin is unknown.

Recognition, Relay, and Elimination

PRL signals through the binding-induced dimerization of single transmembrane receptors that activate various kinases including its canonical Janus kinase 2 (JAK2)-signal transducer and activator of transcription (STAT) pathway, the phosphatidylinositol 3-kinase (PI3K)/AKT pathway, and the mitogen-activated protein kinase (MAPK) pathway (11). Of note, PRL is produced by endothelial cells (24, 109) and uses the JAK2-STAT5 pathway to promote their migration and tube formation (83, 109) and to stimulate the expression of proangiogenic factors (FGF-2 and VEGF) by various nonendothelial cells (40, 41), suggesting that PRL acts not only as a systemic, but also as an autocrine/paracrine positive regulator of angiogenesis. On the other hand, vasoinhibins inhibit angiogenesis, vasodilation, and vasopermeability by mechanisms that include blocking the activation of the Ras-Raf-MAPK pathway, the Ras-Tiam1-Rac1-Pak1 pathway, and the Ca^{2+}/calmodulin-activation of endothelial nitric oxide synthase (eNOS) (46). They also promote protein phosphatase 2A-induced dephosphorylation/inactivation of eNOS (44), the activation of proapoptotic proteins of the Bcl-2 family, and the nuclear factor-κB (NFκB)-mediated activation of caspases. These and other intracellular signaling pathways of vasoinhibins have been reviewed recently (22, 23). The exact nature of the mechanism by which vasoinhibins interact with their target cells to activate these signaling pathways is unclear. However, the discovery of high affinity vasoinhibin binding sites on bovine brain capillary endothelial cells was reported in 1992 (26). These vasoinhibin-binding sites have a high binding affinity, are saturable, and specific. Cross-linking experiments identified 52-kDa and 32-kDa proteins as the major vasoinhibin binding species. However, until today, the exact identity of these vasoinhibin putative receptors has not been revealed.

A target (66) and frequent binding partner of vasoinhibins is plasminogen activator inhibitor-1 (PAI-1). PAI-1 is a serine protease inhibitor that by inhibiting tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA) blocks the generation of plasmin from plasminogen and suppresses clot dissolution (36). PAI-1 forms a complex with uPA and uPAR on endothelial cell membranes (12). Of note, vasoinhibins upregulate PAI-1 expression in endothelial cells (66) and were recently reported to form a complex with endogenous PAI-1 in the culture medium of these cells, as well as in human and mouse plasma (6). Vasoinhibins also colocalized with the PAI-1-uPA-uPAR complex on the endothelial cell surface and formation of such multiprotein complex was required for the antiangiogenic activity of vasoinhibins. This was concluded from experiments demonstrating that silencing or pharmacological blockage of PAI-1 and uPAR signaling abrogated the antiangiogenic effect of the vasoinhibins in HUVEC (6). Of note, the apparent molecular weight of the binding proteins (52 kDa and 32 kDa) of the above-mentioned high affinity vasoinhibin binding site (26) is close to the molecular masses of PAI-1 (43 kDa), uPA (51 kDa), and uPA receptor (55 kDa), and their identity could indeed be the PAI-1-uPA-uPAR complex. However, the specific binding affinity of vasoinhibins to these endothelial membrane species was much higher (K_d = 10 nM) than to PAI-1 (1 μM) or uPA-PAI-1 (0.5 μM). While the difference in affinity may suggest a higher K_d for the natural multicomponent PAI-1-uPA-uPA receptor complex, the contribution of other vasoinhibin binding sites, for example a new cell surface receptor, cannot be disregarded and continues to be an unresolved question.

A role further downstream in the vasoinhibin-signaling cascade, downstream from a potential cell surface receptor or signaling via PAI-1-uPA-uPAR, is described for the vasoinhibin-miR146a (microRNA-146a) circuit. It appears from the studies in PPCM models, that vasoinhibins mediate part of their antiangiogenic effects via induction of miR146a in endothelial cells. If exposed to vasoinhibins, HUVEC proliferation is reduced via upregulation of miR146a and HUVEC release miR146a-loaded exosomes that can be absorbed by neonatal rat cardiomyocytes (51). In these cardiomyocytes, miR146a has a detrimental effect and reduces cardiomyocyte metabolic activity, an effect that presumably contributes to the pathogenesis of PPCM (51). Both anti-miR146a transfection and silencing or pharmacological blockage of PAI-1 and uPAR signaling result in an abrogation of the vasoinhibin antiangiogenic effect. This implies that one of these signals has to be regulated by the other, and further studies are required to characterize this regulatory loop. The other two prominent vascular effects of vasoinhibins besides inhibition of angiogenesis, inhibition of vasodilation, and vasopermeability are effects mediated by vasoinhibin-induced blockage of eNOS activity (44, 46). It remains to be shown whether vasoinhibins utilize the miR146a-circuit and/or interaction with PAI-1 to downregulate eNOS activation.

No data exist about the half-life of vasoinhibins in the circulation. The extent as to which the liver and the kidney contribute to the clearance of vasoinhibins is also unknown. However, vasoinhibins were detected in the urine of women with preeclampsia (47, 65), pointing toward the possibility of an altered renal elimination of vasoinhibins under pathophysiological conditions.

New Physiological and Clinical Implications

New physiological and clinical implications of the prolactin/vasoinhibin axis arise from the governing principle of the growth-restricting effects of vasoinhibins. Vasoinhibins mediate their growth-restricting effects via the inhibition of angiogenesis, which reduces tissue growth due to the lowering of oxygen and nutrient availability. Physiological tissue growth and angiogenesis is fundamental in pregnancy, where angiogenesis is a prerequisite for growth of the placenta and therefore fetal growth.

During pregnancy, PRL level progressively rise to ~20–40 ng/ml at the end of the first trimester, 50–150 ng/ml by the end of

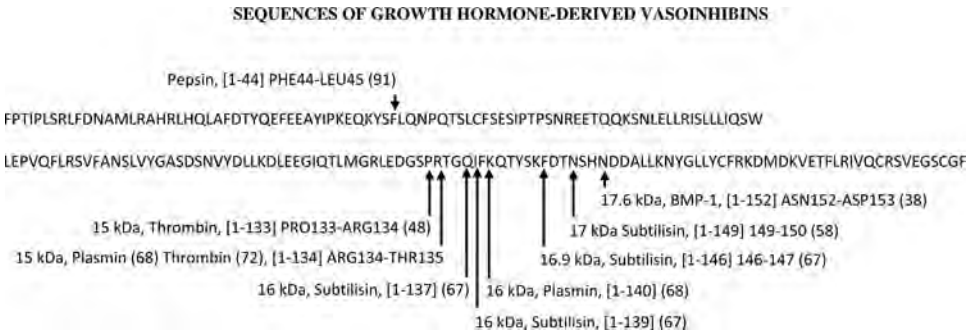

Fig. 3. The protein sequences of full-length growth hormone (GH) and of GH-derived vasoinhibins are presented, including information on their molecular mass and enzyme involved in the vasoinhibin generation and cleavage sites (indicated with arrows). The number in brackets indicate the residues comprising the respective vasoinhibin molecule. None of these GH-related vasoinhibins have been tested for anti-angiogenic activity.

the second trimester, and 100–400 ng/ml at term (100). This rise in circulating PRL level is accompanied by a rise in circulating vasoinhibin level (87), perhaps due to the increased PRL availability for pituitary vasoinhibin generation and release (73). Another source of PRL during pregnancy is the decidual tissue, especially at the maternal-fetal interface (76). While the known physiological purpose of the increase in PRL levels includes the preparation of the breast for lactation, the physiological effect of the increase in vasoinhibin level is less clear, particularly since an elevation of vasoinhibin levels is associated with diseased pregnancy states and adverse pregnancy outcomes.

Vasoinhibins are generated in the human placenta with higher rates observed in placentas from women with preeclampsia (70) and Type 1 diabetes mellitus (78). Furthermore, vasoinhibins are elevated in serum, urine, and amniotic fluid of women with preeclampsia compared with normotensive controls, and their occurrence in urine increases with the severity of the disease and the occurrence of adverse outcomes (47, 64, 65). In fact, the presence of vasoinhibins in urine predicts adverse maternal and perinatal outcomes. The odds ratio in women with preeclampsia and urinary vasoinhibins for combined adverse maternal outcomes (pulmonary edema, acute renal failure, placental abruption, hepatic hematoma or rupture, intubation required, and use of inotropics) is 44.9 (95% CI 5.1–392.3), for stillbirths or neonatal deaths is 1.3 (95% CI 0.4–3.6), and for small for gestational age infants is 1.9 (95% CI 1.1–3.1) (64). The antiangiogenic effects of vasoinhibins and the paramount importance of an adequate vascularization of the placenta imply a causal link between vasoinhibins and these outcomes. Furthermore, human and rat placental tissue featuring higher vasoinhibin synthesis in the presence of maternal diabetes demonstrates hypovascularization with reduced vascular surfaces and capillary density (78). Also, the level of vasoinhibins in amniotic fluid from patients with preeclampsia is inversely correlated with birth weight, that is, higher vasoinhibin level in the amniotic fluid associates with lower birth weight (47), and vasoinhibins antagonize the proangiogenic actions of VEGF in amniotic fluid from patients with preeclampsia (47).These outcomes as well as the pregnancy-associated diseases of preeclampsia and PPCM share a common milieu of increased inflammation, a factor in the activation of the proteolytic enzyme cathepsin D. Therefore, it is conceivable that a higher placental synthesis of vasoinhibins, combined with their increased pituitary release into the circulation during pregnancy, contributes to abnormal placental vascularization and thus to intrauterine growth restriction and small for gestational age infants. Indeed, the regulation of factors stimulating and inhibiting growth between the maternal organism and the fetoplacental unit is usually resolved at the level of the maternal reproductive tract. However, in the fetoplacental unit, a pathophysiological increased synthesis of vasoinhibins may disrupt the balance of growth factors and their inhibitors that contribute to adverse maternal and perinatal outcomes. This disruption is also speculated to occur at the level of organ dysfunction seen in association with increased vasoinhibins, such as the cardiac myocyte [PPCM (54, 55)], the endothelium [preeclampsia (47, 64)], the kidney [preeclampsia (47, 64)], and as above, the placenta (preeclampsia, fetal growth abnormalities). Clinical studies in women with PPCM using bromocriptine to suppress PRL secretion and cardiac vasoinhibin synthesis point toward beneficial effects, and a randomized, multicenter clinical trial is currently under way (49, 50, 53, 88).

Unresolved Questions

Prolactin and growth hormone (GH) are phylogenetically related, they evolved from a common ancestral gene (75), and share a long-chain class-I helical cytokine structure comprising four α-helices. Placental lactogen (PL), the third member of the GH/PRL/PL-family, and another class-I helical cytokine arose independently by duplication of the PRL gene in rodents and ruminants and by duplication of the GH gene in primates (42). GH and PL likely contribute to the generation of endogenous vasoinhibins (Figs. 3 and 4); however, the generation and effects of GH- and PL-derived vasoinhibins have received little attention. Recombinant vasoinhibins derived from GH and PL

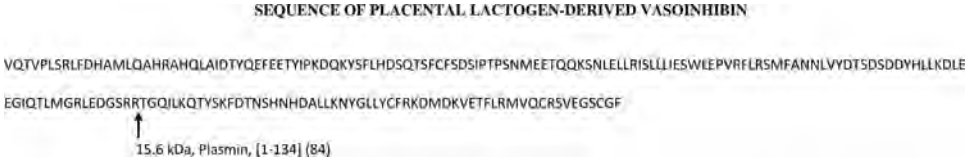

Fig. 4. The protein sequences of full-length placental lactogen (PL) and of PL-derived vasoinhibins are presented, including information on their molecular mass and enzyme involved in the vasoinhibin generation and cleavage site (indicated with arrow). The number in brackets indicate the residues comprising the vasoinhibin-molecule. The PL-derived vasoinhibin has not been tested for anti-angiogenic activity.

displayed antiangiogenic effects in vitro (92). However, no information about their endogenous generation and clinical relevance is available. Importantly, GH-derived, vasoinhibin-like fragments were not detected in the rat pituitary, suggesting that any possible, endogenously GH-derived vasoinhibins may not be generated in the pituitary gland (73). Thus the contribution of GH and PL cleavage resulting in the synthesis of vasoinhibins to the function or regulation of the prolactin/vasoinhibin axis is unclear. The observation that GH is not converted to vasoinhibins in the rat pituitary, suggests that the possible contribution of GH to the total endogenous vasoinhibin level is restricted to the peripheral tissue level. Placental lactogen is increased in pregnancy and is produced by the human placenta. It is not known whether PL is converted to vasoinhibins at the level of the placenta as well.

It is unclear if the differences in molecular mass impact the structure and function of vasoinhibins. Also, it remains to be shown whether and to what extent each of the various proteases contributes to the physiological, endogenous level of vasoinhibins, and how the synthesis of different vasoinhibins is modified in disease conditions. Accordingly, the total composition and full identity of endogenous vasoinhibins in the circulation and at the target-tissue levels, including their interplay, has yet to be determined. The impact of glycosylation of PRL on vasoinhibin physiology is unknown. It is possible, however, that glycosylation alters the proteolytic cleavage of PRL and may thus impact vasoinhibin generation, as well as the action and clearance of these peptides (10). These unresolved questions have delayed the development of a uniform classification/nomenclature for vasoinhibins (97).

Another major unresolved question is the normal concentration of endogenous vasoinhibins in the human circulation. This is owing to the lack of a quantitative assay, with which circulating vasoinhibin levels and reference ranges could swiftly be established. The only method for the evaluation of endogenous vasoinhibins, which is considered relatively reliable is immunoprecipitation and Western blotting. However, this method is only semiquantitative and subject to significant interassay variability. Mass spectrometric techniques were successfully adapted to determine vasoinhibins in sera (106); however, these techniques are experimental and not commercially available. The urgent need for a quantitative assay for vasoinhibins is further underscored by the clinical implications of vasoinhibins in diabetic complications, cancer, and pregnancy-associated diseases, in which the quantitation of circulating vasoinhibins are of relevance for diagnosis, treatment, and risk stratification.

Phylogenetic Context

Prolactin is a pleiotropic hormone whose functions are classified into seven categories: water and electrolyte balance, growth and development, endocrinology and metabolism, brain and behavior, reproduction, immunoregulation and protection, and actions associated with pathological disease states (13). As vasoinhibins are structurally and functionally distinct from PRL, their function is not considered in the classification of PRL biological effects. However, since PRL constitutes the precursor of vasoinhibins, and their regulation and function is embedded into the prolactin/vasoinhibin-axis, the effects of vasoinhibins cannot be comprehended without the consideration of PRL biology. Remarkably, vasoinhibins feature actions in the same categories. It derives from this consideration, that the evolutionary forces that governed PRL phylogenesis may have also determined the emergence of vasoinhibins.

Prolactin, GH, and PL have evolved from a common ancestral gene. On the basis of sequence comparisons of tetrapod hormones, the divergence is located approximately 392 million years ago (29). Two models for the function of the ancestral gene that gave rise to the GH/PRL family have been proposed. The first model proposes that the function of the ancestral gene was to regulate somatic growth (90), while the opposing model proposes that the gene was involved in osmoregulation because this function is common to fish PRL and GH (14). The third member of the somatotropin/prolactin family in fish, besides PRL and GH, is somatolactin (3, 81). The significance of somatolactin as an ancestor of the GH/PRL family in higher vertebrates in the context of the vasoinhibin physiology is not known. However, sequence analyses of somatolactin and human PRL demonstrates a low consensus, and none of the known cleavage sites of human PRL that are required for the generation of vasoinhibins are present in somatolactin.

Since the emergence of the PRL gene from its ancestor, periods of rapid change within the coding sequence of mature PRL lead to substantial sequence differences between human PRL and that of nonprimate mammals and other species (104). There is no evidence for this divergence creating an overall loss of function of PRL in higher primates, making an increased acceptance of mutations associated with a loss of function unlikely (104). Instead, Wallis et al. argue that it is more likely that the periods of rapid change corresponded to periods in which physiological functions of PRL varied and slowing the rate of evolution accompanied a stabilization of the biological function. Furthermore, Wallis et al. (104) propose that the nonrandom distribution of substitutions, coupled with no major loss of function supports positive selection as the most probable explanation for the episodes of rapid evolution. The gain of function of PRL that must have occurred during this process has not been elucidated. However, such gain of function of PRL could have corresponded with the emergence of the ability of PRL to function as a precursor for vasoinhibins. This view is supported when comparing the cleavage sites present in human PRL across taxa. An orthologue comparison with a protein sequence alignment of representative species of several major taxons is presented in Table 2. Indeed, while five cleavage sites are present in the human and the gorilla PRL, four cleavage sites are present in the macaque, three in the pig and the oppossum, two in the duck and in the *Xenopus tropicalis*, and none in the zebrafish and the spotted gar (Table 2). It appears, thus, that vasoinhibins emerged in tetrapods and that throughout speciation, there was an increase in cleavage sites with the highest number of cleavage sites present in primate species. Along this line, the ability of vasoinhibins to balance blood vessel growth, function, and involution could have contributed to PRL effects throughout vertebrate phylogeny and to its value as a biologically conserved factor (1, 20). A limiting factor is that the selected species may represent exceptions rather than representatives of the rule. A full comparative genomics approach will help to strengthen this analysis.

Table 2. *Prolactin cleavage sites required for vasoinhibin generation in different species*

		Protein-Alignment Within Cleavage Site Regions					
		Cathepsin D			MMP	BMP-1	
Taxon	Species	15 kDa Vi	16.8 kDa Vi	17.2 kDa Vi	17.7 kDa Vi	18 kDa Vi	Cleavage Sites
Teleost fishes/*Otophysa*	Zebrafish	. . .GLEHVVHK. . .	. . .LSTLPFNG. . .	. . .STLPFNGN. . .	. . .GNNLGQDK. . .	. . .LGQDKTSR. . .	0
Ray-finned fishes/*Neopterygii*	Spotted gar	. . .GVEKVAEK. . .	. . .SSA- - - -. . .	. . .SA- - - - DA. . .	. . .-DALLPSA. . .	. . .PSSASNDA. . .	0
Lobe-finned fishes/*Sarcopterygii*	Coelacanth	. . .GMECIVGQ. . .	...SELQAPWP. . .	. . .QAPWPG-P. . .	. . .G-PLLLLD. . .	. . .LLLDGEDQ. . .	0
Tetrapods/*Tetrapoda*	*X. tropicalis*	. . .GMEKIVGR. . .	. . .NDVNSLWS. . .	. . .**NSLW-SGPP**. . .	. . .GPMAAQSA. . .	. . .**AQSA-DENS**. . .	2
Reptiles and birds/*Sauria*	Duck	. . .GMEKIVGR. . .	. . .NEIYSQWE. . .	. . .ISQWEGLP. . .	. . .**GLPS-LQLA**. . .	. . .**LQLA-DEDS**. . .	2
Marsupials/*Marsupialia*	Opossum	. . .GMEKIVGQ. . .	. . .NEVYSVWS. . .	. . .**ISWV-SGLP**. . .	. . .**GLPS-LQMA**. . .	. . .**LQMA-DEDT**. . .	3
Laurasiatherian Mammals/ *Laurasiatheria*	Pig	. . .GMEKIVGQ. . .	. . .NEVYSVWS. . .	. . .**YSVW-SGLP**. . .	. . .**GLPS-LQMA**. . .	. . .**LQMA-DEDT**. . .	3
Old World monkeys/ *Cercopithecinae*	Macaque	. . .**GMEL-IVSQ**. . .	. . .**NEIY-PVWT**. . .	. . .YPVWTGLP. . .	. . .**GLPS-LQMA**. . .	. . .**LQMA-DEES**. . .	4
Great apes/*Hominidae*	Gorilla	. . .**GMEL-IVSQ**. . .	. . .**NEIY-PVWS**. . .	. . .**YPVW-SGLP**. . .	. . .**GLSP-LQMA**. . .	. . .**LQMA-DEES**. . .	5
Great apes/*Hominidae*	Human	. . .**GMEL-IVSQ**. . .	. . .**NEIY-PVWS**. . .	. . .**YPVW-SGLP**. . .	. . .**GLSP-LQMA**. . .	. . .**LQMA-DEES**. . .	5

A PRL orthologue comparison using a human PRL protein sequence alignment of representative species of major taxons. The protein sequences were retreived from the ENSEMBL genome browser (http://www.ensembl.org/index.html), release 79, March 2015 (35, 41). The protein sequence alignment shows the regions at which proteolytic cleavage of full-length PRL occurs, resulting in the generation of vasoinhibins (Vi). Regions with cleavage sites are marked in boldface and the cleavage site (marked with -) and the neighboring four NH_2-terminal and COOH-terminal amino acids are shown. No cleavage sites were found in representative species of teleost, Ray-finned, and Lobe-finned fishes. Two cleavage sites are found in the PRL sequence of the *X. tropicalis*, a different set of two cleavage sites in the duck, and three, four, and five cleavage sites are present in representative species of marsupials, Laurasiatherian mammals, Old World monkeys, and Great apes, respectively.

An additional level of complexity, beyond the scope of this review, is the contribution of other members of the PRL family in rodents, such as proliferins and proliferin-related proteins known to have effects on blood vessels (31, 89). This also applies for species-specific GH or PRL family gene expansions in different species including primates, rodents and ruminants (2, 102, 103, 107).

Perspectives and Significance

The process of vasoinhibin generation and secretion from the anterior pituitary gland appears to be under physiological control through mechanisms affecting the biosynthesis of PRL and the expression/activity of PRL cleaving proteases. Also, the circulating PRL levels and the local synthesis of PRL affect the peripheral generation of vasoinhibins at the target tissue level. Therefore, the generation, secretion, and regulation of vasoinhibin action are embedded into the organizational principle of an axis, which integrates the hypothalamus, the pituitary, and the target tissue microenvironment. On the basis of the established nomenclature for other endocrine axes, this axis is designated as the prolactin/vasoinhibin axis. The significance of the prolactin/vasoinhibin axis in pathophysiological states is surfacing to provide a better understanding of the role of PRL in clinically apparent disease conditions. Among these, diseases associated with the state of pregnancy are most prominent as profound changes in PRL metabolism during pregnancy and postpartum seem to render the prolactin/vasoinhibin axis particularly susceptible for disturbances that would cause major changes in the production/action of vasoinhibins. Recent studies concerned with the search for vasoinhibin signaling mechanisms, and particularly with the cell surface receptor involved in their actions, are providing valuable insights into the complexity of vasoinhibin actions within the vascular and nonvascular microenvironment.

This review brings into focus major research challenges needed to advance the field in the near and mediate future. A systematic approach is required to help identify the physiologically relevant vasoinhibin molecules and the cleaving proteases that generate them. This approach should include the characterization through uniform conditions of their antiangiogenic, antivasopermeability, antivasodilatatory effects, and signaling mechanisms. This knowledge will help develop a proper nomenclature for the definition of the various endogenous vasoinhibin isoforms and the development of a quantitative assay capable of differentiating them and their precursor proteins (PRL, GH, PL) in body fluids. The latter is a major demand for studying the involvement of vasoinhibins in pregnancy-associated diseases, diabetic complications, and cancer and for their implications in risk stratification, diagnosis, and treatment. Naturally, resolving the solution structure of vasoinhibins by NMR spectroscopy, possibly by analyzing recombinant human vasoinhibins, would enhance our understanding of the structure-function relationships of the vasoinhibin family and produce valuable insights into their mechanism of action. Upon successful resolution of these issues, the knowledge of the role of vasoinhibins in health and disease will be greatly enhanced and the development of a sustainable classification of vasoinhibins that provides orientation in future biomedical research, will commence.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS

Author contributions: J.T. and C.C. conception and design of research; J.T., D.R.-B., C.F.P., and C.C. analyzed data; J.T. and C.C. interpreted results of experiments; J.T., T.B., D.R.-B., G.M.d.l.E., and C.C. prepared figures; J.T. drafted manuscript; J.T., T.B., D.R.-B., C.F.P., M.H., G.M.d.l.E., and C.C. edited and revised manuscript; J.T., T.B., C.B., D.R.-B., C.F.P., M.H., G.M.d.l.E., and C.C. approved final version of manuscript.

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