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Neonatal Stress: Effects of Hypoglycemia and Hypoxia on Adrenal Tyrosine Hydroxylase Gene Expression

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ABSTRACT

Catecholamines (CA) are released from and resynthesized in the adrenal medulla in response to stress. In the mature animal, stimulus-secretion-synthesis coupling occurs through transsynaptic (neuronal) activity. In contrast, in the immature animal, before functional adrenal innervation, certain stressors (hypoglycemia and glycopenia) do not result in CA release. Additionally, it is not known whether release and biosynthesis remain coupled in the neonate as they are in the adult. Therefore, to evaluate whether neonatal stressors can induce CA biosynthesis at the genomic level "directly" before function adrenal innervation, we studied the expression of the tyrosine hydroxylase (TH) gene, the rate-limiting enzyme in CA biosynthesis. Newborn rat pups were made either hypoxic, hypoglycemic, or cellularly glycopenic (2-deoxyglucose). Neither hypoxic stress nor insulin-induced hypoglycemic stress altered steady state levels of TH mRNA in the neonate. However, cellular glycopenia resulted in a significant 2-fold rise in TH mRNA levels ($p < 0.05$). As expected, each of these stressors increased TH mRNA levels in the mature adult rat. Thus, neonatal hypoxia and hypoglycemia appear to require intact neurogenic impulse activity, whereas cellular glycopenia may "directly" induce TH RNA, perhaps through hormonal mechanisms. This developmental model allows for the analysis of mechanisms governing adrenal CA release separate from those governing biosynthesis at the level of TH RNA. Acute neonatal hypoxic stress results in adrenal CA release without increasing TH RNA. Intrauterine growth retardation from chronic prenatal hypoxemia results in neonatal CA depletion and decreased CA responsiveness. We speculate that chronic hypoxia alters CA pathways, increasing the susceptibility of these infants to later stressors. (***Pediatr Res* 36: 719–723, 1994**)

Abbreviations
TH, tyrosine hydroxylase
2-DG, 2-deoxyglucose
CA, catecholamine

Adrenomedullary release of CA are important mediators of two common stressors that compromise the neonatal transition to extrauterine life: hypoxia and hypoglycemia (1–5). In the absence of the adrenal medulla, the fetus and newborn show maladaptive responses resulting in increased morbidity and mortality (6–8). Because functional innervation of the rat adrenal medulla occurs after the first postnatal week of life (9–11), a developmental window exists whereby the direct effects of stressors can be examined, independent of neuronal impulse activity.

Hypoxia, insulin-induced hypoglycemia, and 2-DG–induced glycopenia act transsynaptically to release adrenal CA in the adult (12–15). On the other hand, in the neonatal rat during the first week of life, hypoglycemic stress fails to result in significant CA release, as presynaptic nerve terminals are nonfunctional at that time (10–12, 14, 16). In contrast, at this same age neonatal hypoxia releases CA through a nonneurogenic mechanism, acting "directly" on the adrenal medulla (11, 14, 17–19). This suggests that the dual roles of transsynaptic activity (regulation of transmitter release and regulation of transmitter biosynthesis) may be functionally separated in the neonate. In the adult, cold stress-induced CA release (and thus depletion of CA stores) results in new CA biosynthesis by activating TH enzyme activity and by increasing the levels of TH mRNA (20, 21). Because neonates do not have functional synapses, we sought to determine whether the stressors hypoxia, hypoglycemia, or cellular glycopenia could directly affect CA biosynthesis by changing levels of TH mRNA at a time when transsynaptic neurogenic mechanisms were nonfunctional. We hypothesized that only hypoxic stress would result in an increase in TH mRNA because it is the only

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one of these stressors that may act directly on the adrenal medulla to release significant amounts of CA in the neonate (14, 18, 19). Surprisingly, we found that TH gene expression was not affected by hypoxic stress, indicating that hypoxia-induced release (nonneurogenic) and biosynthesis could be functionally separated in the first postnatal week of life. As expected, hypoglycemia (a neurogenic-dependent response) did not augment TH mRNA levels in the absence of functional nerve terminals. Unexpectedly, cellular glycopenia did result in increased neonatal TH mRNA levels. These differences may help characterize the basis of adrenomedullary signal transduction mechanisms affecting stimulus-secretion-synthesis coupling and may help explain the maladaptive responses reported in neonates with asphyxia, hypoxia, and intrauterine growth retardation.

METHODS

Animal treatments. After a protocol of Seidler and Slotkin (14, 19), 4-d-old Sprague-Dawley rat pups from two litters were randomized to two groups of 10: hypoxia or room-air controls. For 2 h, all pups were separated from their dams, maintained at 25°C, and exposed to either room air or 0.07 to 0.08 fractional inspired oxygen by a constant flow of nitrogen gas. All pups were returned to their assigned dams and decapitated 24 h later. Medullae were microdissected as previously described (22) and total RNA extracted as previously described (22, 23).

To induce hypoglycemia, 4-d-old rat pups from three litters were randomized to one of three groups of 10: control unmanipulated (*To*), saline vehicle, or insulin as described by us (22) and Lau *et al.* (12). Rat pups were treated with 20 U/kg s.c. of regular insulin (Novolin, Novo Nordisk, NJ) or an equal volume of saline vehicle and placed back with their dams after 2 h as we previously described (22). This treatment resulted in significant neonatal hypoglycemia (12, 16). The pups were decapitated 24 h after injection, adrenal medullae were microdissected, and total RNA was extracted. For comparison, 15 adult male Sprague-Dawley rats weighing 150 to 200 g were fasted overnight and treated with either nothing, saline vehicle, or 10 U regular insulin as we and others previously described (22, 24). After 2 h, insulin-treated rats were rescued with 40% sucrose solution, 2 mL intraperitoneally and 1 mL orally. Animals were dissected 24 and 48 h later and medullae were isolated for RNA extraction.

To induce cellular glycopenia, 4-d-old rat pups from three litters were randomized to three groups of 10: control unmanipulated (*To*), saline vehicle, or 2-deoxyglucose (2-DG, Sigma Chemicals, MO) 500 mg/kg s.c. At this dosage, 2-DG causes a centrally mediated sympathetic stimulation and CA release in mature rats (13, 15, 21). All rats were decapitated 24 h after injection, medullae were microdissected, and total RNA was extracted for Northern analysis. For comparison, 15 adult male Sprague-Dawley rats were fasted overnight and treated with either nothing, saline vehicle, or 2-DG 500 mg/kg and allowed to eat after 2 h as we previously described (22). No resuscitation was necessary for these rats. All animals were killed 24 h later, and adrenal medullae were removed for RNA extraction.

Molecular techniques. Total RNA was extracted using minor modifications of the acid guanididium thiocyanate phenol chloroform method (23, 25) and quantified by UV spectroscopy as previously described (22). Ten micrograms of total RNA were fractionated on a glyoxal denaturing gel, electrotransferred to a nylon filter, UV crosslinked, baked for 2 h at 80°C, and prehybridized overnight (22). Prehybridizing and hybridizing conditions were as previously described (22). After washing and autoradiography, the image intensities were quantified as previously described (22).

A *Kpn*I-*Pst*I 282 bp fragment of the plasmid $pTH._4$ [kindly provided by E. Lewis, Oregon University, Portland, OR (26), and which contains the coding region for the rat TH gene] was radiolabeled with [α^{32}P]dCTP to a sp act of $>10^9$ cpm/μg using standard random primer kits (Boehringer-Mannheim, IN, or Stratagene, CA) (22). As an RNA-loading control, filters were reprobed to the constitutively expressed gene for glyceraldehyde 3-phosphate dehydrogenase, using a *Pst*I 1269-bp fragment of the plasmid pR-glyceraldehyde 3-phosphate dehydrogenase-13 (provided by K. Marcu, State University of New York, Stony Brook, NY) (27).

Statistics. Each of these experiments was performed three times. A *t* test was performed in analyzing the hypoxia experiments. Analysis of variance followed by Neuman-Keuls test was used for the insulin and 2-DG experiments. A p value of <0.05 was considered significant (28).

RESULTS

Exposing neonatal rat pups to hypoxia for 2 h, a treatment that results in CA release directly through a nonneurogenic mechanism, did not result in increased adrenal TH mRNA levels, as seen in Figure 1. Neonatal pups treated with insulin to induce hypoglycemia, a treatment that results in no significant release of adrenal CA at this age (it requires functional transsynaptic activity), also exhibited no resulting increase in TH mRNA levels, as seen in Figure 2. When pups were made glycopenic with 2-DG, a treatment that also results in no significant release of adrenal CA, the result was a 2-fold rise in TH mRNA levels ($p < 0.05$), as seen in Figure 3. In contrast to these studies in pups, when mature rats are exposed to these stressors, TH mRNA increased as expected 1.5-fold after insulin shock at 24 h and 1.7-fold after 48 h ($p < 0.05$ *versus* control and saline), as seen in Figure 4. Adult rats treated with 2-DG had TH mRNA levels 2.2 times that of controls ($p < 0.05$). Similar results were obtained when using either 18S ribosomal RNA as a loading control or expressing the results as TH RNA per total RNA loaded.

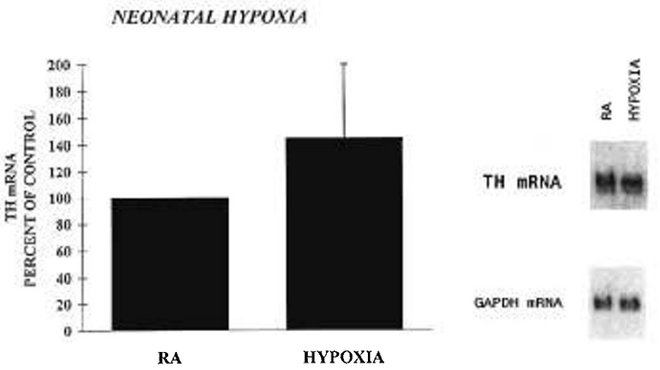

Figure 1. Effect of neonatal hypoxia on TH gene expression. Newborn rat pups were exposed to hypoxia (0.07 to 0.08 fraction of inspired oxygen) for 2 h at 4 d of life (at a time before functional innervation to the adrenal medulla). Although this stress results in a significant release of catecholamines, there was no significant increase in adrenal TH mRNA levels as determined by Northern blot analysis compared with room-air (*RA*) controls.

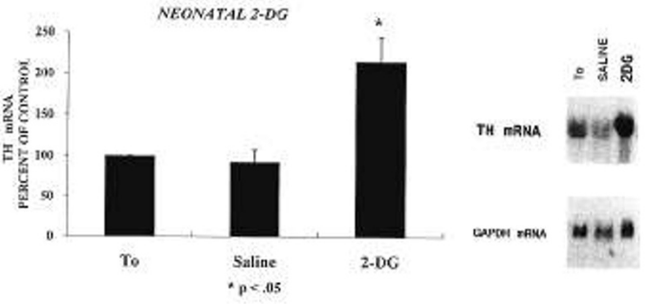

Figure 3. Effects of neonatal glycopenia on TH gene expression. Neonatal rats treated with 2-DG results in no significant catecholamine release, but Northern analysis shows a significant 2-fold rise ($p < 0.05$) in TH mRNA compared with unmanipulated controls (*To*) or saline vehicle.

DISCUSSION

CA are essential for the successful transition to extrauterine life (6–8, 18, 29). At birth, the CA surge is largely due to their release from the adrenal medulla (8, 29) and occurs primarily in response to hypoxic stress and other factors active at delivery (7, 8, 18, 29). As innervation to the adrenal medulla is incomplete in the rat fetus and newborn, hypoxic stress results in CA release "directly" through nonneurogenic mechanisms (8, 9, 18). This nonneurogenic release may be a direct effect of hypoxia on the chromaffin cells, or it may be acting through hormones released from this stress. For example, prolactin, corticosteroids, and angiotensin II each have been shown to regulate CA release in cultured fetal chromaffin cells (30). Later, after functional innervation to the adrenal medulla is complete, these nonneurogenic mechanisms are no longer active, and hypoxia results in CA release by a centrally mediated sympathetic innervation [as ganglionic blockade with chlorosondamine prevents this release (8, 14)]. In contrast, insulin-induced hypoglycemia and 2-DG–induced glycopenia result in adrenal CA release only in the presence of functional innervation, as this response is a centrally mediated transsynaptic event.

Although neonatal hypoxia results in CA release "directly" (through a nonneurogenic mechanism), we found no increase in steady state TH mRNA levels in the adrenal medulla. This suggests that there was an uncoupling of release and biosynthetic mechanisms at this age, at least at the level of TH RNA. Alternatively, hypoxia may have resulted in TH activation without increasing

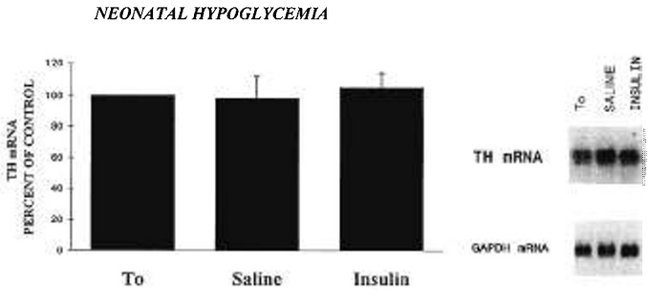

Figure 2. Effects of neonatal hypoglycemia on TH gene expression. Insulin-induced hypoglycemia in neonatal rats results in no significant catecholamine release, and Northern analysis shows no significant difference in adrenal TH mRNA levels compared with unmanipulated controls (*To*) or saline vehicle.

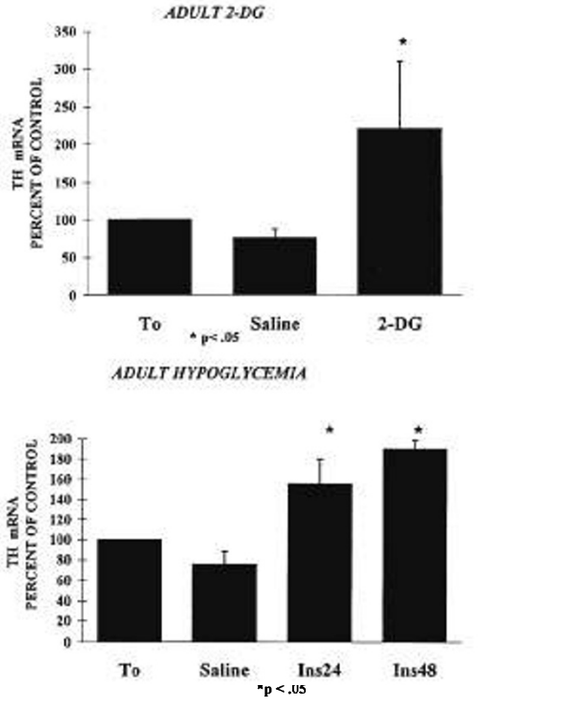

Figure 4. Effects of glycopenic and hypoglycemic stress on TH gene expression in the adult rat. *Top*, Adult rats were made glycopenic with 2-DG and controls treated with saline vehicle. TH mRNA was significantly elevated ($p < 0.05$) after 2-DG treatment, compared with saline vehicle or unmanipulated control (*To*) *Bottom*, A single hypoglycemic stress in the adult rat resulted in a significant ($p < 0.05$) elevation in TH mRNA levels 24 and 48 h after insulin injection, compared with saline vehicle and unmanipulated control (*To*). There was no difference between TH mRNA levels at 24 and 48 h after insulin injection. Hypoxemia was previously reported to have no significant change in adrenal TH mRNA levels in the adult rat (31).

TH RNA; however, we did not measure TH protein or TH activity. Moreover, it is well recognized that the neonatal rat pup can respond to cholinergic receptor agonists (8, 19) with release of CA, increased adrenal TH activity (8), and enkephalin content (31), consistent with functional extracellular receptors coupled to intracellular cholinergic-linked second-messenger systems. Ultimately, hypoxia-induced CA release without new biosynthesis would represent a maladaptive response, as the net result would be CA depletion.

The release of CA from the adrenal have been shown to be critical for the neonatal adaptive response to postnatal stress and, in particular, for pups to survive hypoxic stress (7, 8, 14, 18, 29). For example, adrenalectomized rats that are hypoxia-stressed have a greater mortality (70 *versus* 20% control), independent of steroid replacement, illustrating the critical need for CA (6). In the case of the chronically stressed intrauterine growth-retardation model of Wigglesworth (32), CA release and biosynthetic mechanisms appear to be deficient. In the intrauterine growth-retardation–model animal, CA release is eventually attenuated in response to acute hypoxia and biosynthesis is not stimulated as compared with control (17), making these animals more vulnerable to postnatal stress. This would suggest that chronic prenatal hypoxic stress has significant long-term postnatal effects on CA pathways.

In the adult adrenal, hypoxia results in increased TH activation (33) but does not increase in TH mRNA (34). On the other hand, in the adult rat carotid body, hypoxia acts directly to increase TH mRNA in a dose-responsive manner (34) and also augments CA biosynthesis (35). The TH gene may well have a hypoxia-sensitive element with a consensus sequence similar to that of the erythropoietin gene (36) that responds to low Po_2 in the carotid body and PC12 (rat pheochromocytoma) cells. Because the Po_2 in the fetus is very low already, alterations in TH gene expression in response to hypoxia in the neonate may be dampened, or the response may be tissue-specific or cell-specific (34).

In hypoglycemic stress, Khalil *et al.* (3) have described a two-stage response in the adult rat. The first part is neurogenic (early), whereas the second part (late) involves a nonneurogenic mechanism that could not be blocked by cholinergic antagonists (3). This nonneurogenic late component occurred when the serum blood sugar level fell below 75 mg/dL and may represent a hormonal response (3). In the neonatal rat, as expected, before functional innervation, we found that transsynaptically mediated hypoglycemic stress failed to alter CA biosynthesis at the level of TH mRNA (Fig. 2). Using this protocol (12, 16), the level of hypoglycemia was well below the threshold described by Khalil (75 mg/dL). Thus, this late, nonneurogenic pathway may also be unresponsive or nonfunctional in the neonatal rat pup exposed to hypoglycemia.

Interestingly, in contrast to insulin-induced hypoglycemia, 2-DG–induced cellular glycopenia, which does not result in CA release in the newborn, did result in a significant 2-fold rise in TH mRNA levels (Fig. 3). Thus, cellular glycopenic stress appears to be sufficient to induce TH gene expression at a time before functional neuronal innervation. Alternatively, because 2-DG treatment results in high serum glucose levels and insulin treatment in low glucose levels, the hormonal responses differ. Together, the data best support the conclusion that 2-DG is nonneurogenic and probably a hormonal response or may be some other direct pharmacologic effect of 2-DG. In any case, neonatal 2-DG treatment may be useful as a model to separate nonneurogenic or hormonal mechanisms that result in increased TH mRNA (*e.g.* biosynthesis) independent of those neurogenic (transsynaptic) stimuli that result in CA release.

In adult rats, hypoglycemic stress results in TH activation (20, 21) and increased TH mRNA for at least 2 d after the stress (Fig. 4). Glycopenia with 2-DG, which is known to increase sympathetic drive, was also associated with increased TH mRNA in the adult. This effect may also have a nonneurogenic component as well which may be hormonal (*e.g.* glucagon, etc.). Glucocorticoids are unlikely to be involved, as each of these stressors result in corticosteroid release in the neonate as well as in the adult (15, 38). Thus, it is doubtful that any increase in TH mRNA after 2-DG treatment can be attributed to glucocorticoids alone; other hormonal mechanisms are likely to be involved.

Our data suggest that the newborn rat is better suited to respond (or adapt) to acute hypoxic stress by CA release than to hypoglycemia (neither CA release nor biosynthesis). This may represent a teleologic adaptation, as the birthing process frequently results in a transient hypoxemia and very infrequently in hypoglycemia. The corresponding period of noninnervated adrenal medulla in humans is approximately 23 wk (39), suggesting that the first- and second-trimester fetus may be particularly vulnerable to the direct effects of hypoglycemic stress.

In summary, we found that stressors that induce CA release and biosynthesis in the adult rat do not induce CA pathways in parallel in the neonate. Neonatal hypoxia results in CA release, but not in CA biosynthesis at the level of TH RNA. Only neonatal glycopenia resulted in a significant rise in TH mRNA. We speculate that these nonneurogenic mechanisms are hormone-based and that the newborn rat offers a developmental model in which to access the direct effects of stressors on the adrenal medulla, independent of neurogenic mechanisms, and that using this paradigm one may study mechanisms of release separate from biosynthesis.

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