Regulation of Catecholamines by Sustained and Intermittent Hypoxia in Neuroendocrine Cells and Sympathetic Neurons

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Abstract—Chronic intermittent hypoxia, a characteristic feature of sleep-disordered breathing, induces hypertension through augmented sympathetic nerve activity and requires the presence of functional carotid body arterial chemoreceptors. In contrast, chronic sustained hypoxia does not alter blood pressure. We therefore analyzed the biosynthetic pathways of catecholamines in peripheral nervous system structures involved in the pathogenesis of intermittent hypoxia-induced hypertension, namely, carotid bodies, superior cervical ganglia, and adrenal glands. Rats were exposed to either intermittent hypoxia (90 seconds of room air alternating with 90 seconds of 10% O₂) or to sustained hypoxia (10% O₂) for 1 to 30 days. Dopamine, norepinephrine, epinephrine, dihydroxyphenylacetic acid, and 5-hydroxytryptamine contents were measured by high-performance liquid chromatography. Expression of tyrosine hydroxylase and its phosphorylated forms, dopamine β-hydroxylase, phenylethanolamine N-methyltransferase, and GTP cyclohydrolase-1 were determined by Western blot analyses. Both sustained and intermittent hypoxia significantly increased dopamine and norepinephrine content in carotid bodies but not in sympathetic ganglia or adrenal glands. In carotid bodies, both types of hypoxia augmented total levels of tyrosine hydroxylase protein and its phosphorylation on serines 19, 31, 40, as well as levels of GTP cyclohydrolase-1. However, the effects of intermittent hypoxia on catecholaminergic pathways were significantly smaller and delayed than those induced by sustained hypoxia. Thus, attenuated induction of catecholaminergic phenotype by intermittent hypoxia in carotid body may play a role in development of hypertension associated with sleep-disordered breathing. The effects of both types of hypoxia on expression of catecholaminergic enzymes in superior cervical neurons and adrenal glands were transient and small.

Key Words: blood pressure □ catecholamines □ chemoreceptors □ sympathetic nervous system □ sleep apnea syndromes □ oxygen □ adrenal gland

Sleep-disordered breathing (SDB) is a frequent condition that affects ≈5% of the population and is characterized by repetitive episodes of hypoxemia and hypercapnia, followed by arousal, respiratory recovery, and reoxygenation. Persistent SDB is causally associated with substantial cardiovascular morbidity, the most important being systemic arterial hypertension (for recent comprehensive review, see Fletcher, 2003). The mechanisms involved in the genesis of this hypertension appear to involve several different components including augmented sympathetic nerve activity,1–4 altered function of arterial chemoreceptors,5–7 elevated levels of circulating norepinephrine (NE),8–10 decreased vascular responses to nitric oxide,11 increased plasma concentrations of endothelin,12,13 and altered regulation of the renal kallikrein-kallistatin pathway.14 The use of animal model systems in which different components of SDB can be studied individually have revealed that intermittent hypoxia (IH) is the critical stimulus underlying development of increased sympathetic activity and hypertension.15,16 Furthermore, the effect of IH requires the presence of functional arterial chemoreceptors.1,5–7

The O₂-sensitive cells (type I) of the carotid bodies (CB) closely monitor O₂ tension in the arterial blood and, through release of various neurotransmitters, deliver afferent neural traffic information to the respiratory and cardiovascular networks in the brain stem, thereby triggering hyperventilation and increased sympathetic nerve activity. The predominant neurotransmitters synthesized and released in CB type I cells, in a manner highly correlated with the level and duration of hypoxia, are the catecholamines dopamine...
(DA)\(^{17-19}\) and NE.\(^{19}\) Both DA and NE regulate CB function through feedback attenuation of hypoxia-activated calcium conductance, inhibiting calcium-activated intracellular signaling pathways and reducing the release of other neurotransmitters.\(^{20,21}\) Both inhibit hypoxia-induced ventilation.\(^{22,23}\)

Our laboratory previously demonstrated that in CB type I cells, sustained hypoxia (SH) induces gene expression for tyrosine hydroxylase (TH) (EC 1.14.16.2), the rate-limiting enzyme in catecholamine biosynthesis.\(^{24}\) The hypoxic induction of TH mRNA contributes to increased enzymatic activity of the TH protein, which leads to augmented synthesis and release of DA. Using an O\(_2\)-sensitive cell line (PC12), which serves as a convenient model system, we found that the increase in TH gene expression during hypoxia is due to augmented TH gene transcription and increased stability of TH mRNA.\(^{25}\) In contrast, hypoxia did not affect expression of the TH gene in sympathetic neurons or adrenal chromaffin cells.\(^{24}\)

The enzymatic activity of TH is positively regulated by its phosphorylation.\(^{26-29}\) Four phosphorylation sites have been identified: Ser8, Ser19, Ser31, and Ser40. Phosphorylation of the last three serines is regulated in vivo\(^{30}\) and modulates TH activity. Phosphorylation of Ser40 by protein kinase A (PKA),\(^{28,29}\) calcium/calmodulin-dependent protein kinase II (CaMK II),\(^{31}\) or mitogen-activated protein kinase-activated protein (MAPKAP) kinase-1 or -2\(^{31}\) increases TH activity, resulting from the loss of inhibition by catecholamines.\(^{32}\) Phosphorylation of Ser31 by MAP kinases also activates TH\(^{33}\) Ser19 is phosphorylated by CaMKII, and this appears to increase the rate of phosphorylation of Ser40.\(^{34}\) However, little is currently known about the effects of hypoxia on the phosphorylation of TH. Although hypoxia elicits membrane depolarization and increases intracellular calcium in PC12 cells,\(^{35}\) both of which may augment TH phosphorylation, hypoxia also inhibits the activities of some of the TH phosphorylating enzymes such as CaMKII and PKA.\(^{36}\)

The essential cofactor for TH activity, (6R)-tetrahydrobiopterin, is synthesized de novo from GTP by GTP cyclohydrolase-I (GTPCH-I, EC 3.5.4.16), the first and rate-limiting step of this biosynthetic pathway.\(^{37}\) It is currently not known if the expression or activity of this enzyme is subject to regulation by O\(_2\).

The present study was undertaken to evaluate possible alterations in the peripheral catecholaminergic system during development of arterial hypertension associated with SDB through the use of the well-characterized rat IH experimental model system.\(^{14,38,39}\) We assessed catecholamine biosynthesis in neuroendocrine cells of CB and adrenal gland (AG) and in sympathetic neurons of superior cervical ganglia (SCG) of rats exposed to IH (which become hypertensive) and of rats exposed to SH (which remain normotensive). We found that both SH and IH increase DA and NE content, TH protein and phosphorylation levels, and GTPCH-I protein levels in the CBs. However, the magnitude of such changes was markedly smaller during IH as compared with SH. Furthermore, no major changes were observed in catecholamine biosynthesis in sympathetic neurons of the SCG or in AG cells during either SH or IH.

### Methods

#### Materials

The following antibodies were used: polyclonal anti-TH (1:10,000) and anti-phenylethanolamine N-methyltransferase (PNMT; 1:5000) from Protos Biotech Corp, monoclonal anti-dopamine \(\beta\)-hydroxylase (DBH) (1:500) from Pharmingen (BD Biosciences), and anti-TH-Ser40-P (1:5000) from Cell Signaling Technology. Phospho-specific antibodies against TH phosphorylated on Ser31 and Ser19 were as previously described\(^{40}\) and used at a concentration of 0.8 \(\mu\)g/mL in 1% polyvinylpyrrolidone, 0.05% Tween-20. The polyclonal antibody against GTPCH-I (1:500) was a generous gift from Dr G. Kapatos (Wayne State University, Detroit). Secondary HRP-linked anti-rabbit and anti-mouse immunoglobulins were used.

#### Animals

Male rats (Sprague Dawley; 175 to 200 g) were exposed to either IH or SH for 1, 3, 7, 14, or 30 days in commercially designed chambers (Oxybox model A44X0, Biospherix) as described previously.\(^{14,38,39}\) Blood pressure was measured with a telemetry system (Data Sciences International). The transmitters (TA11PA-C40) were implanted at least 7 days before recordings began.

#### Preparation of Protein Extracts and Immunoblotting

Frozen tissues were homogenized in RIPA buffer (10 mmol/L Tris-HCl pH 7.5, 300 mmol/L NaCl, 3 mmol/L MgCl\(_2\), 0.5% NP40, 0.1% SDS, 0.5% sodium deoxycholate, 0.5 mmol/L PMSF, 10 mmol/L NaF, 10 \(\mu\)g/mL leupeptin, 10 \(\mu\)g/mL aprotinin, 1 \(\mu\)mol/L lactacystin, 2 mmol/L orthovanadate) with a Dounce homogenizer (pestle B) at 4°C. The homogenates were incubated at 4°C for 1 hour with gentle agitation and centrifuged at 15,000 rpm for 20 minutes. The supernatants were further centrifuged at 40,000 rpm for 30 minutes.

Equal amounts of lysates (80 \(\mu\)g of CB extracts and 150 \(\mu\)g of AG or SCG extracts) were denatured by boiling in SDS sample buffer, resolved by SDS-PAGE on 4% to 22% gradient gels, and detected by immunoblotting as previously described.\(^{41}\) The data are presented as percent changes in immunoreactivity in hypoxic samples as compared with the immunoreactivity measured in normoxic samples in the same experiment and on the same blot.

#### High-Performance Liquid Chromatography Measurements of Catecholamines

Frozen tissues were homogenized in 0.2N perchloric acid and centrifuged at 13,000 rpm for 15 minutes. NE and epinephrine (E) were quantified in a 20-\(\mu\)L sample injected onto a C-18 column (MD-150, ESA, Inc) connected to a LC-4B amperometric detector (Bioanalytical Systems) and a glassy carbon electrode with a potential of +600 mV. DA, dihydroxyphenylacetic acid (DOPAC), 5-hydroxytryptamine (5-HT) were quantified in samples injected onto a C-18 column (MD-150, ESA, Inc) connected to a Coullochem detector (ESA, Inc), The values are given as mean±SEM. Analysis was performed by GraphPad Instat version 3.0 for Windows, with 1-way ANOVA, followed by Tukey-Kramer multiple comparison tests. Differences having a value of \(P<0.05\) were considered to be statistically significant.

An expanded Methods section can be found in an online supplement available at http://www.hypertensionaha.org.

#### Statistical Evaluation of the Data

Data are expressed as mean±SEM. Analysis was performed by GraphPad Instat version 3.0 for Windows, with 1-way ANOVA, followed by Tukey-Kramer multiple comparison tests. Differences having a value of \(P<0.05\) were considered to be statistically significant.

#### Results

**Blood Pressure**

Rats exposed to IH developed significantly higher values of systolic and diastolic blood pressure as compared with
animals exposed to room air or SH (Figure 1). The increase in systolic and diastolic blood pressure started within the first week of exposure, and both values were significantly higher (\(P < 0.001\)) at the end of 30 days of exposure as compared with animals exposed to room air.

Carotid Body

Exposure of rats to 10% SH for 14 days increased DA and NE content measured in CBs (Figures 2A and 2B). Equivalent durations of IH increased catecholamine content as well; however, the effects of IH were significantly smaller than those mediated in response to SH (Figure 2). Both types of hypoxia resulted in similar increases in the concentration of the DA metabolite DOPAC (Figure 2C). In contrast to catecholamines, hypoxia did not affect the concentration of another biogenic amine, serotonin (5-HT) (Figure 2D).

Consistent with the above results, SH strongly increased the levels of TH immunoreactivity, as well as that of phosphorylated TH sites (Figures 3 and 4). The increases in total TH protein accumulation and phosphorylations on Ser19, Ser31, Ser40 residues developed rapidly and occurred within the first day of exposure to SH.

IH also affected TH protein accumulation and phosphorylation in CBs. However, the increases in TH expression and phosphorylation were not as prominent as those induced by SH (Figures 3 and 4). Furthermore, the increase in TH protein accumulation was delayed during IH and began only after 7 days of exposure, such that the final increase measured at the end of the 30 days’ exposure to IH was 5-fold smaller than that elicited by corresponding exposures to SH (Figures 3 and 4). Similarly, phosphorylation of TH protein during IH was less prominent and delayed until days 14 and 30 days of exposure (Figure 3). The effects of SH or IH on DBH levels in the CB were only transient (Figure 3), and no PNMT immunoreactivity was detected in CB extracts (not shown).

Superior Cervical Ganglia and Adrenal Glands

In contrast to the substantial effects of both SH and IH on catecholamine metabolism in CB tissues, no significant changes occurred in the SCGs and AGs. The Table shows measurements of DA, NE, E, DOPAC , and 5-HT contents in sympathetic neurons and adrenal chromaffin cells. The absence of significant differences in catecholamine content was consistent with the absence of major changes in TH protein content or phosphorylation (Figure 5).

The effects of sustained and intermittent hypoxia on immunoreactivity of TH protein and its phosphorylation in SCGs and AGs were small compared with the changes measured in CB, and the differences were not statistically significant (Figure 5). In SCGs, exposure to SH resulted in a decrease in the immunoreactivity for phospho-Ser19 or -Ser40 and DBH and a very small increase in total TH protein.

Figure 1. Effects of exposure to sustained or intermittent hypoxia for the indicated number of days on systolic (●) or diastolic (○) blood pressure values. Data are expressed as average blood pressure ± SEM (n=6). At day 30, blood pressure levels were significantly higher in rats exposed to intermittent hypoxia than to sustained hypoxia or room air (**\(P < 0.001\)).

Figure 2. Effects of 14 days of sustained (SH) and intermittent (IH) hypoxia on CB DA (A), NE (B), DOPAC (C), and 5-HT (D) levels. Mean±SEM values are shown. *\(P < 0.05\), **\(P < 0.01\), ***\(P < 0.001\) (normoxic samples, n=6; hypoxic samples n=15).
and phospho-Ser31 (Figure 5A). Exposure to IH resulted in somewhat stronger inducing effects on the total TH protein and phosphorylation on Ser19 and Ser31. Immunoreactivity of phospho-Ser40 first increased and then decreased. DBH levels showed some level of induction that was transient in SH and more long-lasting in IH (Figure 5A).

In AGs, SH modestly increased TH protein levels, particularly at the later time points, and had no statistically significant effects on the pattern of TH phosphorylation (Figure 5B). Phosphorylation on Ser19 was somewhat increased (not significantly) in response to 30 days of SH, whereas phospho-Ser31 and phospho-Ser40 immunoreactivities were transiently increased around days 3 to 7 and then decreased to control levels by 30 days of hypoxic exposure. IH induced transient increases in TH protein, phospho-Ser31, and phospho-Ser40 levels between days 3 and 7. However, no changes in TH phosphorylation occurred on Ser19 in the AG extracts of rats exposed to IH (Figure 5B). Furthermore, both types of hypoxia induced DBH protein levels, and neither SH or IH had any effect on PNMT protein levels in the AG.

GTP Cyclohydrolase-1

To evaluate the potential role of the tetrahydrobiopterin cofactor in regulation of TH activity by hypoxia, we assessed the effects of hypoxia on GTPCH-1, the rate-limiting enzyme in tetrahydrobiopterin synthesis, in carotid bodies (Figure 6). SH induced significant accumulation of GTPCH-1 protein, beginning at day 1 of exposure and remaining elevated for the entire duration of this stimulus. IH also increased GTPCH-1 protein levels, but this response was delayed, and levels after 14 days of IH were comparable to those induced by 24 hours of SH (Figure 6). Thus, accumulation of GTPCH-1 was not affected in SCGs or AGs after either SH or IH (Figure 6).

Discussion

Rats without any genetic hypertensive background exposed to our chronic IH protocol had elevated systolic and diastolic blood pressure, thereby confirming previous studies using a variety of intermittent hypoxia protocols. In this context, we examined markers of the catecholamine biosynthetic pathway in peripheral catecholaminergic tissues di-
directly participating in adaptation to hypoxia and in development of arterial hypertension during SDB. We made several novel observations, providing insights into mechanisms by which hypoxia induces catecholamine biosynthesis in CBs. For the first time, we show that hypoxia induces phosphorylation of TH at serines 19, 31, and 40 in CB cells and also increases GTPCH-1 protein levels in this organ. These results extend previous work from this laboratory and demonstrate that the hypoxic induction of TH protein and DA biosynthesis is regulated at multiple steps, including induction of gene expression, posttranslational modifications, and induction of the biosynthesis of the relevant cofactors.

Although IH and SH result in similar levels of low arterial oxygen tension (mean $\text{PaO}_2$ = 40 to 42 mm Hg; D. Gozal, personal communication, 2002), IH produces much smaller increases in DA and NE, TH protein, TH phosphorylation, and GTPCH-1 protein levels. This lesser induction of the catecholaminergic pathway by IH versus SH holds true whether total or absolute durations of exposure (ie, total exposure time or actual time spent in hypoxia, respectively) are considered. Indeed, 30 days of exposure to IH increased TH protein levels and regulated its phosphorylation state to a similar degree as only 1 day of SH. Clearly, however, the absolute hypoxic exposure during 30 days of IH was longer than that during 1 day of SH.
than during 1 day of SH. A possible mechanistic explanation is that IH, alternating episodes of hypoxia and reoxygenation, causes oxidative stress.\textsuperscript{39} TH expression is induced by hypoxia\textsuperscript{24,25,41} but is downregulated by oxidative stress.\textsuperscript{42} At the same time, mechanism(s) responsible for DA turnover appear to be affected similarly by both SH and IH. Indeed, the levels of DOPAC, the DA metabolite generated by monoamine oxidase, are similar in CBs from rats exposed to either SH or IH. The increases in NE concentration in the CB appear to correlate with the levels of DA and thus may simply result from increased amount of substrate for DBH, the expression of which is not altered by hypoxia in the CB. However, an increase in the number of DBH-expressing glomus cells, as proposed by others,\textsuperscript{43} needs to be considered.

The data provide important insights in understanding the role of CB in development of IH induced hypertension. The smaller induction of catecholamine expression in CB during IH as compared with SH may contribute to the relatively higher sympathetic response during IH. Both DA and NE have attenuating effect on the CB activity and inhibit hypoxia-induced hyperventilation.\textsuperscript{20–23} Thus, it is likely that the lower amounts of DA and NE released during IH are less attenuating, leading to much a stronger activation of CB input, which is then translated into higher sympathetic activity.

Relatively high levels of NE induced by both types of hypoxia in CB are comparable with the levels measured in SCGs, and thus CB could be a source of elevated blood NE. Such a strong effect of carotid body on plasma catecholamine levels is not unlikely in view of known circulatory effects of biogenic amines released from CB tumors or other small paragangliomas.\textsuperscript{44,45} Yet, SH stimulates higher amounts of NE synthesized (and released from) by the CB than IH, without any decrease in blood pressure. This finding indicates that both CB and IH have different effects on peripheral vascular mechanisms, the former promoting vasodilatation and the latter promoting vasoconstriction. This is consistent with impairment of the kallikrein-kallistatin pathway, vasodilatation, and vascular hyper trophy in response to IH but not to SH.\textsuperscript{14}

An intriguing observation is that neither SH or IH altered catecholamine content in SCGs or AGs and that the effects on expression of catecholamine synthesizing enzymes were very small. These results differ somewhat from the earlier reports showing an increase in TH protein in AGs from rats exposed to SH for up to 22 days\textsuperscript{46} and in DA but not NE in SCGs.\textsuperscript{47} A possible explanation for this discrepancy is that catecholamine contents in these studies were expressed per individual ganglion or gland, the size of which increases with chronic hypoxia, whereas our measurements were normalized to protein content in each tissue. The increases in TH $V_{\text{max}}$ have been previously reported in sympathetic postganglionic neurons and cells of adrenal medulla (see references in Czyzynski-Krzeska et al, 1992\textsuperscript{24}) in response to relatively short-lasting SH. However, we failed to detect any changes in TH total protein levels or its phosphorylation during the first day of exposure to sustained hypoxia in SCGs and measured a weak increase in total TH protein in AG. A potential explanation is that hypoxia causes an early and transient increase in TH enzymatic activity that disappears within 24 hours of exposure, the time frame at which we began our measurements. Interestingly, we observed a small tendency for increase in DBH levels in SCG and AG during IH but not SH. Although this increase in DBH did not correlate with increased NE levels in SCG and AG, the results suggest that perhaps longer exposures to IH would elevate NE in these tissues through augmented DBH activity.

The results also suggest that IH could lead to increased sympathetic activity by inducing molecular changes in the upstream central nervous system neurons without any changes in the neurotransmitter biosynthesis in the peripheral neurons. In accordance with this hypothesis, chronic IH stimulates persistent expression of immediate early genes in the brain stem.\textsuperscript{48}

Perspectives

Hypertension occurring during SDB results from SDB-associated IH and requires activity of arterial chemoreceptors. We have provided initial evidence that attenuation of catecholamine biosynthesis and release in CB during IH as compared with SH may contribute to augmented chemoreceptor input and increased sympathetic output leading to the development of hypertension. In addition, we found limited evidence to support any major effects of IH on catecholaminergic phenotype of AG or SCG.

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References


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