Activation of tyrosine hydroxylase by intermittent hypoxia: involvement of serine phosphorylation

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Activation of tyrosine hydroxylase by intermittent hypoxia: involvement of serine phosphorylation. A novel mechanism of intermittent hypoxia (IH) that activates tyrosine hydroxylase (TH) was investigated in rat pheochromocytoma 12 (PC-12) cells by exposing them to alternating cycles of hypoxia (1% O₂, 15 s) and normoxia (21% O₂, 3 min) for up to 60 cycles; controls were exposed to normoxia for a similar duration. IH exposure increased dopamine content and TH activity by ∼42 and ∼56%, respectively. Immunoblot analysis revealed that comparable levels of TH protein were expressed in normoxic and IH cells. Removal of TH-bound catecholamines and in vitro phosphorylation of TH in cell-free extracts by the catalytic subunit of protein kinase A (PKA) increased TH activity in normoxic but not in IH cells, suggesting possible induction of TH phosphorylation and removal of endogenous inhibition of TH by IH. To assess the role of serine phosphorylation in IH-induced TH activation, TH immunoprecipitates and extracts derived from normoxic and IH cells were probed with anti-phosphoserine and anti-phospho-TH (Ser-40) antibody, respectively. Compared with normoxic cells, total serine and Ser-40-specific phosphorylation of TH were increased in IH cells. IH-induced activation of TH and the increase in total serine and Ser-40-specific phosphorylation of TH were inhibited by Ca²⁺/calmodulin-dependent protein kinase (CaMK) and PKA-specific inhibitors but not by inhibitors of the extracellular signal-regulated protein kinase pathway, suggesting that IH activates TH in PC-12 cells via phosphorylation of serine residues including Ser-40, in part, by CaMK and PKA. Our results also suggest that IH-induced phosphorylation of TH facilitates the removal of endogenous inhibition of TH, leading to increased synthesis of dopamine.

IN HUMANS, INTERMITTENT HYPOXIA (IH) associated with recurrent apneas leads to the development of pathophysiological conditions such as hypertension (9–11), whereas chronic sustained hypoxia, as it occurs at high-altitude dwelling, does not result in such adverse effects. The pathological consequences of intermittent but not chronic sustained hypoxia led us to hypothesize that IH activates mechanisms that are distinct from those of chronic sustained hypoxia. To begin to define these cellular mechanisms coupled to IH, we developed a method (1) that permits the exposure of cell cultures to alternating cycles of hypoxia and normoxia, simulating the pattern of hypoxic episodes seen in recurrent apnea (28, 29). For this mechanistic investigation, we opted to examine the effect of IH on the activity of tyrosine hydroxylase (TH) in pheochromocytoma 12 (PC-12) cells.

PC-12 cells, an oxygen-responsive cell line, are often used as a neuronal model to examine cellular mechanisms associated with hypoxia (20, 25, 37, 41). These cells express TH activity and synthesize and store catecholamines (13, 23). TH (EC 1.14.16.2; tyrosine 3-monoxygenase) is the rate-limiting enzyme in catecholamine biosynthesis (21) that catalyzes the conversion of tyrosine into dihydroxyphenylalanine (DOPA). Both molecular oxygen and tetrahydrobiopterin are required for enzyme activity. Extensive information pertaining to the mechanism of in vivo regulation of TH is available. For instance, the activity of TH in vivo is regulated by site-specific phosphorylation involving specific protein kinases (2, 14, 16, 36, 38–40). The NH₂-terminal region of TH contains four serine residues (Ser-8, Ser-19, Ser-31, and Ser-40) that undergo phosphorylation in response to a variety of physiological stimuli (6, 12, 15, 16, 19, 22, 32, 40), generating a more active form of the enzyme. Thus, in PC-12 cells, depolarization increased TH activity by two- to threefold, and this activation is due to increased extracellular signal-regulated kinase (ERK)-dependent and CAM-dependent protein kinase A (PKA)-dependent phosphorylation of the enzyme at Ser-31 and Ser-40 (15, 32). Furthermore, the activity of TH is augmented by chronic sustained hypoxia involving increased expression of TH proteins (27, 33, 35).

In this study, we exploited these regulatory features of TH along with the oxygen-sensitive, cellular responses of PC-12 cells to characterize the cellular effects of IH. After cell cultures were exposed to alternating cycles of brief hypoxia and normoxia, enzyme activity, protein expression, and serine phosphorylation were measured.

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tion of TH were determined. Our results suggest that IH increases TH activity in PC-12 cells by mechanism(s) involving increased serine phosphorylation without augmenting TH protein expression.

MATERIALS AND METHODS

Cell cultures. PC-12 cells (original clone from Dr. L. Green) were cultured on polystyrene-coated Falcon 100-mm culture dishes (BD-Falcon Biosciences, Lexington, TN) in a humidified chamber maintained with 5% CO₂ and 21% O₂ at 37°C as described previously (20). The growth medium (DMEM) was supplemented with 10% horse serum, 5% fetal calf serum, and 100 μM of sodium penicillin G. The medium was replaced once in 2 days. The results reported in this study were obtained from cells maintained between passages 4 and 9, and all experiments were performed in serum-free medium.

Materials and reagents. Tyrosine, catalase, (6R)-5,6,7,8-tetrahydrobiopterin, DMSO, okadaic acid, and penicillin G were purchased from Sigma Chemical (St. Louis, MO). KN-62, KN-93, PD-98059, PKA inhibitor-α (14-22) amide peptide (Myr-N-Gly-Arg-Thr-Gly-Arg-Asn-Ala-Ile-NH₂), phosphatase inhibitor cocktail set I (containing 2.5 mM (-)-p-bromotetramisole oxalate, 500 μM cantharidin and 500 nM microcystin-LR), and forskolin were obtained from Calbiochem (La Jolla, CA). PKA catalytic subunit was from New England Biolab (Beverly, MA). Anti-TH monoclonal antibody was from Pel-Freez (Rogers, AK). Anti-TH polyclonal antibody and anti-phospho-TH (Ser-40) antibody were obtained from Cell Signaling Biotechnology (Beverly, MA). Anti-phosphoserine polyclonal antibody was from Chemicon International (Temecula, CA). Protein A-agarose was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Complete protease inhibitor cocktail (EDTA-free) tablets were from Roche. Stock solutions of drugs, with the exception of antibodies and enzymes, were prepared in DMSO and diluted into appropriate buffers for experiments.

Exposure of cells to IH. For exposure of cells to IH, cell cultures were placed in a humidified Lucite chamber. The culture medium was continuously monitored with a molecular oxygen electrode (Lazar), respectively, and recorded on a strip chart recorder. During hypoxic challenge, a period of 45 s was required for ambient oxygen to reach 1% O₂; during normoxia, a period of 50 s was required for returning to 21% O₂. Typically, the ambient O₂ of the chamber and the medium was 110 and 70 Torr, respectively. At each cycle of hypoxic exposure, the P0₂ of the chamber and the medium dropped to 20 and 50 Torr, respectively. During IH exposure, the pH of the culture medium remained at 7.4. Cells exposed to either normoxia or intermittent normoxia (i.e., cells placed in the same Lucite chamber but challenged with alternating cycles of 21% O₂ and 1% O₂) served as controls.

Cell viability. To assess the effect of IH on cell viability, lactate dehydrogenase (LDH) activity in the medium was determined spectrophotometrically as described previously (20). Briefly, after exposure to either normoxia or IH, cells were separated from the medium by centrifugation (500 g for 5 min). The LDH activity of the medium was determined by monitoring pyruvate-dependent oxidation of NADH and expressed as micromoles of NADH oxidized per minute per milligram of protein.

Assay of TH activity. The activity of TH was determined by a modification of a method that was previously described (26). Briefly, cells (~3 x 10⁶) were homogenized in 50 mM Tris-HCl buffer, pH 7.4, containing 0.25 M sucrose, complete protease inhibitor cocktail (Roche), and phosphatase inhibitor cocktail set I (Calbiochem) in a glass homogenizer. For activity measurements, the reaction medium (250 μl) consisted of the following components at the final concentrations indicated: 160 mM sodium acetate, 160 mM L-tirosine, 80 μM 6,7,8-tetrahydrobiopterin dihydrochloride in 2-mercaptoethanol, 1.6 μM catalase, and 100 μl of cell homogenate. The pH of the reaction medium was 6.5. After 10–60 min of incubation at 37°C, the reaction was stopped by the addition of 1 M perchloric acid containing 0.2 M EDTA at 4°C. A known amount of freshly prepared DHBA, an internal standard, was added to the reaction medium to assess the efficiency of DOPA recovery. DOPA along with DHBA was extracted from the reaction medium by using acid-activated aluminum oxide as described previously (20) and quantitated by HPLC-ECD as described under Analysis of catecholamine content (above). On average, ~78% DOPA was recovered during extraction, and all data were corrected for recovery losses during extraction and HPLC analysis. TH activity was expressed as the amount of DOPA formed from tyrosine per minute per milligram of protein.

Immunoblot analysis. Extracts of cells exposed to either normoxia or IH, after normalization of protein content, were subjected to SDS-PAGE. For the analysis of protein expression and serine phosphorylation, a range of prestained protein molecular weight standards was included. Proteins were transferred electrophoretically to polyvinylidene difluoride membrane by using a transfer buffer containing 25 mM Tris, 192 mM glycine, 20% methanol, and 0.1% SDS. The efficiency of transfer was verified by Ponceau S staining. Nonspecific binding sites on the membrane were blocked by incubation for 1 h with 5% milk in Tris-buffered saline containing 0.1% Tween 20. Blots were then incubated with a suitable primary antibody solution [such as monoclonal anti-TH or polyclonal anti-phospho TH (Ser-40) or polyclonal anti-phosphoserine antibodies] for 1 h at room temperature followed by several washes with Tris-buffered saline containing 0.1% Tween 20. The blots were then incubated with either horseradish per-
oxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG for 1 h followed by six 10-min washes with Tris-buffered saline containing 0.1% Tween 20. Antibody-labeled proteins were identified with the enhanced chemiluminescence Western blotting detection kit according to the manufacturer’s recommendations (Amersham Biosciences). Autoradiograms were analyzed by scanning densitometry (Kodak Image Station 440CF), and the values were expressed as percentages of normoxic control.

Immunoprecipitation of TH. Typically, cell-free extracts containing 100 µg of protein were incubated with 2 µg of anti-TH monoclonal antibody (Pel-Freez) in 50 mM Tris·HCl buffer, pH 7.4, containing complete protease inhibitor (Roche) and phosphatase inhibitor cocktail set I (Calbiochem) at 4°C overnight. Forty microliters of protein A-agarose (Santa Cruz) suspension were added to the reaction mixture and allowed to incubate at 4°C for 4 h. The agarose beads were collected by centrifugation and washed six to eight times with ice-cold Tris-buffered saline. Under this condition, TH was quantitatively immunoprecipitated from both the control and IH cells as evidenced by either enzyme activity measurements or immunoblot analysis of the supernatant using a polyclonal anti-TH antibody. The immunoprecipitated proteins were resolved by SDS-PAGE and then analyzed for serine phosphorylation of TH by immunolabeling with anti-phosphoserine polyclonal antibody (Chemicon). The immunoblots were developed and quantitated by procedures as described above. The data were corrected for variation, if any, in the amount of TH in the immunoprecipitates derived from various experiments. The amount of total TH proteins (i.e., both phosphorylated and unphosphorylated forms of TH) in the immunoprecipitates was determined by immunoblot analysis with polyclonal anti-TH antibody.

Removal of catecholamines from cell-free extracts. TH protein-bound and free catecholamines in the cell extracts of normoxic and IH cells were removed by gel permeation chromatography. One hundred microliters of cell-free extracts containing ~300 µg proteins were applied on top of a Sephadex G-25 (fine) column (6 × 1 cm) and pre-equilibrated with 50 mM Tris·HCl buffer, pH 7.4, containing complete protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail set I (Calbiochem). The proteins were eluted with 10 ml of the equilibration buffer, and 200-µl aliquots of fractions were collected. The concentration of proteins in the fractions was determined by use of the procedures described below. Protein-containing fractions were pooled, concentrated by Amicon filtration, and used for enzyme activity measurements.

Protein measurement. Protein concentration was determined by the bichinchoninic acid method, using bovine serum albumin as the standard (34).

Exposure of cells to forskolin and protein kinase inhibitors. To determine the contribution of specific protein kinases in the modulation of TH activity by IH, cells (~3 × 10^6) were incubated in Krebs–Ringer buffer medium containing either forskolin or cell-permeable protein kinase inhibitors at the final concentrations indicated. To assess the role of PKA, forskolin (100 nM), an activator of adenylyl cyclase, and PKA inhibitor-α (14–22) amide (Myr-N-Gly-Arg-Thr-Gly-Arg-Asn-Ala-Ile-NH₂; 1 µM) were used. Also, KN-93 (15 µM) and PD-98059 (30 µM) were used to inhibit Ca^2+ /calmodulin-dependent protein kinase (CaMK) and ERK, respectively. In additional experiments, the effect of KN-62, another potent inhibitor of CaMK, was also examined at a final concentration of 10 µM. Either forskolin or protein kinase inhibitor was added to cell culture medium 15 min before IH exposure and remained in the medium during the entire duration of IH exposure. Cells exposed to normoxic medium containing forskolin or protein kinase inhibitor served as controls. After treatments, cell-free extracts were prepared and used for the analysis of TH activity and assessment of serine phosphorylation of TH as described above.

In vitro phosphorylation of TH in cell-free extracts by the catalytic subunit of PKA. Both control and IH cells (~3 × 10^6 each) were extracted in 50 mM Tris·HCl buffer, pH 7.4, containing 0.25 M sucrose, complete protease inhibitor cocktail (Roche), and phosphatase inhibitor cocktail set I (Calbiochem). Cell-free extracts were incubated in the presence of either 25 or 50 units of the catalytic subunit (40 kDa) of PKA in 40 mM HEPES buffer, pH 7.0, containing 10 mM MgCl₂ and 0.5 mM each of ATP, EDTA, and EGTA for 15 min at 25°C. This procedure leads to the phosphorylation and activation of TH (31). For control samples, the extracts were incubated with buffer containing MgCl₂, ATP, EDTA, and EGTA only.

Data analysis. All data presented reflect observations from at least three independent experiments and are expressed as means ± SE. Statistical significance was evaluated by a paired t-test or one-way ANOVA for repeated measures. P values < 0.05 were considered significant.

RESULTS

Cell viability. PC-12 cells were exposed to either normoxia or 60 episodes of IH, and LDH activity in the medium and in the cell pellet was determined to assess cell viability. As shown in Table 1, IH exposure did not significantly alter LDH activity either in the medium or in the cells (P > 0.05, n = 4), suggesting that cell viability was unaffected by IH.

IH and TH activity. TH activity in cell extracts was determined by monitoring the formation of DOPA from l-tyrosine using the HPLC-ECD method. In the control, normoxic cells, TH activity increased linearly with incubation time and reached a plateau between 45 and 60 min of incubation (Fig. 1). A similar dependence of TH activity on incubation time was also seen with IH cells. In subsequent studies, 15-min incubation time was used for the assay of TH activity.

In normoxic cells, TH activity, on average, was 180.1 ± 0.9 pmol·min⁻¹·mg protein⁻¹, and it was higher in cells exposed to IH (Fig. 2A). The magnitude of IH-induced increase in TH activity was dependent on the number of episodes of IH. Thus TH activity increased by ~28% (P < 0.01, n = 6; Fig. 2A) and ~56% (P < 0.01, n = 6; Figs. 1 and 2A) after conditioning the cells with 30 and 60 episodes of IH, respectively. On the other hand, TH activity in cells exposed to 15 episodes of IH remained the same as that of control cells (Fig. 2A). Moreover, increasing the duration of IH from 60 to 120 cycles did not further augment TH activity.

<table>
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<tr>
<th>Sample</th>
<th>Normoxia</th>
<th>IH</th>
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<tbody>
<tr>
<td>Cell pellet</td>
<td>12.96 ± 0.41</td>
<td>13.33 ± 0.68</td>
</tr>
<tr>
<td>Medium</td>
<td>0.53 ± 0.15</td>
<td>0.65 ± 0.12</td>
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Values are means ± SE; n = 4 experiments. IH, intermittent hypoxia; LDH, lactate dehydrogenase. Differences between normoxic and IH cells were not significant.
activity (data not shown). By contrast, TH activity, in cells exposed either to intermittent normoxia or continuous hypoxia (1% O₂) for 15 min, a duration that corresponds to the cumulative duration of hypoxia during 60 cycles of IH (i.e., 15 s × 60), was nearly the same as that of normoxic cells (Fig. 2C). However, prolonged exposure of cells to continuous hypoxia (60 or 120 min) resulted in a modest increase in TH activity (ranging from 10 to 25%; \( P < 0.05, n = 4 \); Fig. 2C). Taken together, these observations suggest that IH, but not a comparable duration of sustained hypoxia, increases TH activity and that IH is a more potent stimulus of TH activity than sustained hypoxia.

Based on the above results, we selected 60 cycles of IH for the following investigations on the mechanism(s) of IH-induced upregulation of TH activity. Cells exposed to normoxia for similar durations served as the control.

**IH and cellular level of DA.** To assess whether IH-induced elevation in TH activity resulted in a concomitant increase in the synthesis of catecholamines, the concentrations of DA in the normoxic and IH cells were compared. On average, the concentration of DA in normoxic cells was 283.9 ± 7.1 pmol/mg of protein. By contrast, in cells conditioned with 60 episodes of IH, DA concentration increased to 403.2 ± 8.3 pmol/mg protein (\( \sim \)42%; \( P < 0.01, n = 4 \)). This finding lends support to the notion that IH-induced TH activation is functionally coupled to an increase in DA level in PC-12 cells.

**IH and TH protein level.** To determine whether the IH-induced increase in TH activity is due to an increase in TH protein level, cell-free extracts of normoxic and IH cells were resolved on SDS-PAGE and probed with anti-TH antibody. Immunoblot analysis showed a similar level of TH protein in normoxic cells and in cells exposed to 15, 30, or 60 episodes of IH (Fig. 2B). These results suggest that the increase in TH activity by IH is not coupled to a concomitant increase in TH protein level but may occur via other mechanism(s), including enzyme activation.

**Effect of removal of catecholamines on TH activity.** TH has been reported to exist in vivo predominantly in an inactive form due to endproduct inhibition via catecholamine binding (3–5). It is likely that IH-induced activation of TH may result from removal of this endogenous inhibition, resulting in the conversion of an inactive form of enzyme to an active form. To test this possibility, cell-free extracts of normoxic and IH cells were subjected to Sephadex G-25 column chromatography to remove TH-bound catecholamines, if any, and

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Fig. 1. Effect of incubation time on tyrosine hydroxylase (TH) activity in cell-free extracts of pheochromocytoma 12 (PC-12) cells exposed to normoxia (C) and 60 cycles of intermittent hypoxia (IH). Cell extracts containing 100 μg of protein were incubated in TH assay medium at pH 6.5. Suitable aliquot of the reaction medium was removed at regular intervals for dihydroxyphenylalanine (DOPA) analysis as described in MATERIALS AND METHODS. Enzyme activity was measured in triplicate. Average data from 6 independent experiments are shown.

Fig. 2. Duration of IH affects the activity but not the protein level of TH in PC-12 cells. A: cell cultures were exposed to 15, 30, and 60 episodes of IH as described in MATERIALS AND METHODS. Cells maintained under normoxia served as controls. Average data from 6 independent experiments are shown. mg P, milligrams of protein. B: immunoblot analysis of TH protein level during exposure of cells to IH. Equal amounts (40 μg) of proteins from the control and IH-conditioned cell extracts were used for the analysis. Other experimental details are as in A. C: comparison of the effect of intermittent vs. sustained hypoxia on TH activity. Average data from 4 independent experiments are expressed as means ± SE. Other experimental details are as in A. SH, sustained hypoxia; IN, intermittent normoxia. \( *P < 0.05; \) \( **P < 0.01; \) n.s., not significant \( (P > 0.05) \).
TH activity in the protein fractions eluted from the column was determined. TH activity, in the normoxic cell extracts, was 182.5 ± 0.5 pmol·min⁻¹·mg protein⁻¹, whereas after removal of catecholamines the activity increased by 100 pmol·min⁻¹·mg protein⁻¹ (−55%; P < 0.01, n = 6; Fig. 3). By contrast, in IH cell extracts, removal of catecholamines had no significant effect on TH activity (P > 0.05, n = 6), suggesting that IH markedly attenuated the feedback inhibition of TH by catecholamines in PC-12 cells.

Influence of IH on serine and Ser-40-specific phosphorylation of TH. Previous studies have shown that the activity of TH in vivo is regulated via phosphorylation of one or more serine residues occurring at the NH₂-terminal region of TH, including Ser-8, Ser-19, Ser-31, and Ser-40 (6, 12, 15, 16, 19, 22, 32, 40), and also by feedback inhibition through binding of catecholamines (3–5). Among these potential phosphorylation sites, phosphorylation of Ser-40 has been implicated to play a significant role in the activation of TH. Furthermore, phosphorylation reversed the endogenous inhibition of TH by disrupting catecholamine binding (3, 5). We have, therefore, assessed the role of serine phosphorylation in IH-induced activation of TH by comparing the level of phosphorylation of TH between the normoxic and IH cells. TH proteins from cell extracts were immunoprecipitated by using monoclonal anti-TH antibody, and the immunocomplexes were analyzed for total TH and phospho-TH by using polyclonal anti-TH and anti-phosphoserine antibodies, respectively. Immunoblot analysis was also performed on cell-free extracts by using anti-TH and anti-phospho-TH (Ser-40) antibodies. Results from these analyses showed that total serine phosphorylation of TH (Fig. 4A), in general, and phosphorylation of Ser-40 of TH (Fig. 4B), in particular, are significantly increased in cells conditioned with 60 episodes of IH. On average, IH caused −62% and 72% increases (P < 0.01, n = 3) in the total serine phosphorylation (Fig. 4C) and Ser-40-specific phosphorylation of TH (Fig. 4D), respectively. These results suggest that IH facilitates phosphorylation of TH at serine residues, especially that of Ser-40.

Evidence for the involvement of CaMK and PKA in IH-induced activation of TH. In vitro studies have identified several protein kinases, including PKA (7, 16, 24, 32), CaMK (6, 12, 39), and ERK (18, 38) involved in the phosphorylation and subsequent activation of TH. To identify which one of these protein kinases contributes to IH-induced activation of TH, cells were treated with cell-permeable inhibitors that are specific to various protein kinases, before IH exposure. TH activities of these cells were compared with those of untreated controls. As shown in Fig. 5, KN-62 and KN-93, inhibitors of CaMK, attenuated IH-induced activation of TH (−50%; P < 0.01, n = 4). Likewise, PKA inhibitor-α (14–22) peptide, a potent cell-permeable inhibitor of PKA catalytic subunit with a Kᵢ of 98 pM, also decreased IH-induced activation of TH (−40%; P < 0.05, n = 4; Fig. 5). However, PD98059, a specific inhibitor of ERK, had no significant effect on the IH-induced increase in TH activity (P >
Multiple protein kinases participate in the activation of TH by IH. Cell cultures preincubated with specific protein kinase inhibitors for 15 min and then exposed to either normoxia (C) or 60 episodes of IH were used. Untreated cells were used as controls. The following protein kinase inhibitors at concentration indicated in parenthesis were tested: KN-62 (10 μM), KN-93 (15 μM), protein kinase A (PKA) inhibitor-α (14–22) peptide (PKAIP; 1 μM), and PD-98059 (30 μM). TH activity in cell extracts was assayed with the use of procedures described in MATERIALS AND METHODS. Average data from 4 independent experiments are shown. *P < 0.05; **P < 0.01. Values are means ± SE; n = 4 experiments. PKA, protein kinase A.

Table 2. Effect of forskolin on TH activity in normoxic and IH PC-12 cells

<table>
<thead>
<tr>
<th>Sample</th>
<th>TH Activity, pmol · min⁻¹ · mg protein⁻¹</th>
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<tr>
<td>Untreated cells</td>
<td>187.0 ± 7.7</td>
</tr>
<tr>
<td>Cells + forskolin (100 nM)</td>
<td>299.4 ± 10.6</td>
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Values are means ± SE; n = 3 experiments. TH, tyrosine hydroxylase.

Table 3. Effect of the catalytic subunit of PKA on TH activity in cell-free extracts from normoxic and IH PC-12 cells

<table>
<thead>
<tr>
<th>Sample</th>
<th>TH Activity, pmol · min⁻¹ · mg protein⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell-free extract</td>
<td>187.0 ± 7.7</td>
</tr>
<tr>
<td>Cell-free extract + catalytic subunit of PKA (25 U)</td>
<td>228.4 ± 10.7</td>
</tr>
<tr>
<td>Cell-free extract + catalytic subunit of PKA (50 U)</td>
<td>271.0 ± 11.2</td>
</tr>
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Values are means ± SE; n = 4 experiments. PKA, protein kinase A.

Evidence for the involvement of CaMK and PKA in the phosphorylation of TH during IH. We examined the ability of cell-permeable protein kinase inhibitors to attenuate IH-induced phosphorylation of TH. First, the level of serine phosphorylation in TH immunoprecipitates derived from the normoxic and IH cells, preincubated with specific kinase inhibitors, was determined and compared with that of untreated controls. As shown in Fig. 6, IH-induced serine phosphorylation of TH was either totally abolished or markedly attenuated (~75%; P < 0.01, n = 3) by KN-62 and PKA inhibitor-α (14–22) peptide, respectively. However, inhibition of ERK had no significant effect. On the other hand, in normoxic cells, the level of serine phosphorylation of TH was partially attenuated by CaMK inhibition (~25%; P < 0.05, n = 3), whereas no significant effect was seen with inhibitors of PKA and ERK (data not shown). These observations suggest that CaMK and PKA may play critical roles in IH-induced augmentation of serine phosphorylation of TH.

Second, we also examined the level of Ser-40 phosphorylation of TH in normoxic and IH cells, preincubated with selected cell-permeable protein kinase inhibitors, and compared the results with those obtained from untreated controls. Immunoblot analyses of the cell-free extracts using anti-phospho-TH (Ser-40) antibody showed that the inhibitor specific to PKA completely abolishes whereas CaMK inhibition partially reduces IH-induced Ser-40 phosphorylation of TH (Fig. 7). By contrast, treatment with PD-98059 did not sig-
significantly alter Ser-40 phosphorylation of TH in IH cells. However, these protein kinase inhibitors had no significant effect on Ser-40 phosphorylation of TH in normoxic cells (data not shown). Our results suggest that both PKA and CaMK play major roles in IH-induced Ser-40 phosphorylation of TH.

DISCUSSION

In this study, our objectives were to determine whether IH affects TH activity in PC-12 cells and to elucidate the mechanism(s) associated with the modulation of TH activity by IH. Our results demonstrated that IH increases TH activity via mechanism(s) involving enzyme activation. Furthermore, we showed that IH-induced TH activation is, in part, due to increased serine phosphorylation, especially that of Ser-40 mediated by PKA and CaMK and the subsequent removal of endogenous product inhibition of TH via disruption of catecholamine binding.

One of the major findings of this study is that the activity of TH, the rate-limiting enzyme in the biosynthesis of catecholamines, increased after cells were exposed to IH. IH-induced TH activation was associated with a parallel increase in cellular DA level and was dependent on the number of episodes of IH. Thus exposure of cells to 60 cycles of IH increased TH activity maximally. This increase in TH activity was specific for IH because neither intermittent normoxia of similar duration nor 15 min of sustained hypoxia, which corresponds to the cumulative hypoxic duration during 60 cycles of IH, affected TH activity. Consistent with this observation is the finding that only prolonged but not short-term hypoxic exposure affects TH activity in cell cultures and in rat brain stem medullary neurons (8, 27, 35). Although prolonged exposure to hypoxia (60 or 120 min) augmented TH activity in PC-12 cells, the magnitude of the increase was lower than that observed with IH-conditioned cells. These observations suggest that IH is a more potent stimulus than sustained hypoxia in augmenting TH activity.

Furthermore, the IH-induced increase in TH activity was not associated with a parallel increase in TH protein level. This is in sharp contrast to the previously reported effects of chronic sustained hypoxia on TH activity in the rat carotid body and adrenal gland (33) and in catecholaminergic rat brain stem areas (27, 35) wherein the increase in TH activity was, in part, due to a concomitant increase in TH protein level. Taken together, these findings suggest that the mechanism of IH-induced increase in TH activity differs from that of chronic sustained hypoxia and primarily involves activation of TH.

Posttranslational modification of serine residues involving specific protein kinases and feedback inhibition by DOPA and DA are the major cellular mechanisms that contribute to the regulation of TH activity in vivo. The possible contribution of posttranslational mechanism(s) to IH-induced activation of TH was examined by comparing the level of TH phosphorylation between the normoxic and IH cells. Immunoblot analysis of TH immunoprecipitates revealed a substantial increase in serine phosphorylation of TH in cells exposed to IH compared with the normoxic control cells. Previously, in vitro studies have identified Ser-8, Ser-19, Ser-31, and Ser-40 of the NH₂-terminal region of TH as target sites for phosphorylation by a variety of protein kinases (6, 12, 15, 19, 22, 32, 40). Furthermore, immunoblot analysis of cell-free extracts with anti-phospho TH (Ser-40) antibody showed that IH specifically...
augments Ser-40-specific phosphorylation of TH. These results are in accord with the conclusion that IH activates posttranslational mechanism(s) involving phosphorylation of serine residues, including Ser-40 of TH.

TH exists in vivo in an active and inactive form, and the later form primarily arises as a result of binding of catecholamines such as DOPA and DA to the metal center of TH. Therefore, we have examined whether the IH-induced increase in TH activity involves mechanism(s) leading to a possible alteration in the ratio of active vs. inactive forms of TH. The protein-bound as well as the free DOPA and other catecholamines could be removed by gel-permeation chromatography. Analysis of TH activity in cell-free extracts, before and after gel-permeation chromatography, showed that removal of catecholamines selectively increased TH activity only in the control, normoxic cells but not in IH cells. These results support the possibility that the IH-induced increase in TH activity arises from the conversion of an inactive form of TH to an active form via removal of enzyme inhibition mediated by catecholamine binding. In vitro studies have also demonstrated that phosphorylation of TH decreased the affinity of catecholamines toward TH, thereby leading to activation of TH (3–5). It is, therefore, likely that IH, by activating serine phosphorylation, disrupts the feedback inhibition by catecholamines, leading to activation of TH. Additional experiments, however, are necessary to further confirm this possibility.

Multiple kinases are involved in the phosphorylation of TH. For instance, in vitro studies have shown that CaMK phosphorylates both Ser-19 and Ser-40 in vitro (4, 12, 16, 39), whereas PKA phosphorylates Ser-40 exclusively, in vitro (7, 12, 40) and in situ (16, 17). On the other hand, ERK has been shown to phosphorylate Ser-31 both in vitro and in situ (18). Several lines of evidence suggest that an increase in Ser-40 phosphorylation is often associated with an increase in TH activity and catecholamine biosynthesis (17, 24, 32). To establish the identity of the protein kinases that participate in the phosphorylation of TH during IH, we have compared the influence of specific inhibitors of various protein kinases on TH phosphorylation. Exposure of cells to either KN-93 or KN-62, potent and selective inhibitors of CaMK, before IH exposure abolished and partially attenuated IH-induced increases in general serine phosphorylation and Ser-40-specific phosphorylation, respectively. Furthermore, inhibitors specific to CaMK also inhibited IH-induced activation of TH. PC-12 cells have been shown to express the CaMKII isoform (32), and IH markedly increased the activity of CaMKII (Yuan and Prabhakar, unpublished observations) in PC-12 cells. Taken together, these results suggest that CaMKII involves IH-induced upregulation of serine phosphorylation especially that of Ser-40 and contributes to the subsequent activation of TH.

Our results also showed that PKA inhibitor-α(14–22) peptide, a selective and potent inhibitor of PKA, completely abolished the IH-induced increase in Ser-40 phosphorylation of TH and partially attenuated IH-induced activation of TH. These results suggest the possible involvement of PKA in the activation of TH by IH. Consistent with this notion are the findings that exposure of cells to forskolin, an activator of PKA, before IH challenge and in vitro phosphorylation of cell extracts with the catalytic subunit of PKA derived from cells exposed to either normoxia or to PKA inhibitor-α(14–22) peptide before IH treatment resulted in the activation of TH but not in cells conditioned with IH.

On the other hand, PD-98059, an ERK pathway inhibitor, was ineffective in preventing the increase in serine phosphorylation and activation of TH during exposure of cells to IH. Interestingly, in PC-12 cells, depolarization has been shown to increase ERK-mediated and PKA-mediated phosphorylation of Ser-31 and Ser-40, respectively (32). Although both IH and depolarization activate PKA-dependent mechanisms for the stimulation of TH activity, additional protein kinases are activated in a stimulus-specific manner. Thus activation of the ERK pathway is required for TH activation by depolarization, whereas CaMKII-mediated phosphorylation contributes to TH activation by IH. Taken together, these results suggest that both CaMKII and PKA may play major roles in the activation of TH during IH.

By contrast, the hypoxia-induced increase in TH gene expression in PC-12 cells required nearly 6 h of exposure to 5% O2, and this increase in gene expression was inhibited by chelerythrine chloride, a protein kinase C inhibitor, in a dose-dependent manner, suggesting the involvement of a member of the protein kinase C family (30) in this response. The fact that increased TH gene expression by sustained hypoxia was also seen in PKA-deficient PC-12 cells suggests that among the myriad of protein kinases only a subset of them is activated, depending on the type and pattern of hypoxia.

In summary, the above results demonstrate that IH facilitates the activation of TH in PC-12 cells via mechanism(s) involving posttranslational phosphorylation of TH at serine residues, including that of Ser-40, mediated in part by CaMKII and PKA. Furthermore, we showed that, unlike chronic sustained hypoxia, IH-induced TH activation is not causally related to increased TH protein level.

DISCLOSURES

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