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Original Research Communication

Pattern-Specific Sustained Activation of Tyrosine Hydroxylase by Intermittent Hypoxia: Role of Reactive Oxygen Species-Dependent Downregulation of Protein Phosphatase 2A and Upregulation of Protein Kinases

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Abstract

We investigated the role of protein phosphatases (PP) and protein kinases in tyrosine hydroxylase (TH) activation by two patterns of intermittent hypoxia (IH) in rat brainstem. Rats exposed to either IH_{15s} (15 s, 5% O_2; 5 min, 21%O_2) or IH_{90s} (90 s each of 10% O_2 & 21%O_2) for 10 days were used. IH_{15s} but not IH_{90s} caused a robust increase in TH activity, dopamine (DA) level, and TH phosphorylation at Ser-31 and Ser-40 in the medulla but not in the pons. Likewise, IH_{15s} but not IH_{90s} decreased activity and expression of protein phosphatase 2A (PP2A) and increased activity of multiple protein kinases. *In vitro* dephosphorylation with PP2A nearly abolished IH_{15s}-induced increase in TH activity. IH_{15s} increased generation of reactive oxygen species (ROS) in brainstem medullary regions which was nearly threefold higher than that evoked by IH_{90s}. Antioxidants prevented IH_{15s}-induced downregulation of PP2A and increases in multiple protein kinase activity with subsequent reversal of serine phosphorylation of TH, TH activity, and DA to control levels. These findings demonstrate that IH in a pattern-specific manner activates TH involving ROS-mediated sustained increase in TH phosphorylation via downregulation of PP2A and upregulation of protein kinases. *Antioxid Redox Signal* 11, 1777–1789.

Introduction

Humans living at sea level experience intermittent hypoxia (IH) under a variety of conditions, including sleep-disordered breathing manifested as recurrent apneas. Studies in humans and animal models showed that IH associated with recurrent apneas leads to autonomic abnormalities, resulting in cardiorespiratory morbidities (6, 10, 13, 28, 32, 38, 43). Brainstem catecholaminergic neurons have been implicated in the regulation of cardiorespiratory systems (9, 12, 18, 25, 34, 35). Consequently, several studies examined the effects of IH on catecholamine metabolism and transmission. Results from these studies showed that IH increases norepinephrine levels in the hypothalamus (33) and alters catecholaminergic neurotransmission in brainstem regions of rat (19, 26). Furthermore, studies in PC12 cells showed that IH increased the activity of TH, the rate-limiting enzyme in the biosynthesis of catecholamines, via increased serine phosphorylation of TH (27). The signaling mechanisms that underlie IH-induced alterations in TH activity, however, remain to be investigated.

Tyrosine hydroxylase (TH; EC 1.14.16.2) catalyzes the conversion of tyrosine into dihydroxyphenylalanine (DOPA) in a molecular oxygen and tetrahydrobiopterin-dependent manner. TH activity *in vivo* is regulated post-translationally by reversible site-specific serine phosphorylation and therefore depends on the balance between protein kinases-mediated phosphorylation and protein phosphatases-dependent dephosphorylation reactions (1, 4, 11). The NH_2-terminal region of TH contains three serine residues (Ser-19, Ser-31, and Ser-40) and increased phosphorylation of one or more of these serine residues leads to TH activation (5, 15, 22, 31). Protein kinases, including protein kinase A (PKA), Ca^{2+}/calmodulin-dependent kinase II (CaMKII), and extracellular signal-regulated kinase (ERK), phosphorylate TH at one or more serine residues, resulting in a more active form of TH (see

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ref. 11 for references). Available evidence suggests that protein phosphatase 2A (PP2A) is the major serine/threonine phosphatase that dephosphorylates TH at Ser-31 and Ser-40 residues (2, 11, 20), leading to less active form of TH.

Chronic sustained hypoxia increased TH activity in the brainstem which was primarily attributed to upregulation of TH protein (42, 46). On the other hand, studies in PC12 cells (27) showed that IH-induced increase in TH activity is not associated with a concomitant increase in TH protein, suggesting IH-induced TH activation. Contrasting this finding, Gozal *et al.,* (17) using an IH protocol comprising alternating cycles of hypoxia (10% O_2) and normoxia (21% O_2) of 90 s each (*i.e.*, IH_{90s}) reported a lack of stimulatory effect of IH on TH activity. Given the differences in IH protocols used in these two studies (17, 27), it is likely that the stimulatory effect on TH activity by IH is pattern specific. Therefore, in the present study, we investigated whether IH patterns consisting of alternating cycles of 5%O_2 for 15 s and 21% O_2 for 5 min (*i.e.*, IH_{15s}) which is similar to that used in our previous cell culture studies (27), and IH_{90s} affect TH activity and catecholamine level in the rat brainstem regions implicated in cardiorespiratory control. Furthermore, we also examined the signaling mechanisms that underlie the differences in the response of TH activity to IH_{15s} and IH_{90s} by focusing on the roles of reactive oxygen species (ROS), PP2A, and protein kinases in IH-evoked TH activation.

Materials and Methods

The Institutional Animal Care and Use Committee of the University of Chicago approved animal handling and experimental protocols. The studies were performed with adult male Sprague–Dawley rats weighing 200–230 g.

Exposure to two patterns of IH

The rats were exposed to two different patterns of IH. For the first IH pattern (IH_{15s}), the animals were placed in an IH chamber and exposed to alternating cycles of hypoxia (15 s of 5% O_2) and normoxia (5 min of 21% O_2) for 8 h/day between 9:00 AM to 5:00 PM for 10 days, as described previously (40). The second IH protocol (IH_{90s}) consists of alternating cycles of hypoxia (10% O_2) and normoxia (21% O_2) for 90 s each for 10 days, as described previously (17). Ambient O_2 level in the IH chamber was continuously monitored with an O_2 analyzer (Beckman model OM-11). Inspired CO_2 level was maintained at 0.2–0.5% and monitored continuously by an infrared analyzer (Beckman model LB-2). The duration of the gas flow during each hypoxic and normoxic episode was regulated by timed solenoid valves. Animals exposed to normoxia for 10 days served as controls.

In experiments wherein the role of ROS was assessed, rats were given either manganese (III) tetrakis (1-methyl-4-pyridyl) porphyrin pentachloride (MnTMPyP; Alexis Biochemicals, CA; 5 $mg.kg^{-1}.day^{-1}$; I.P.) or *N*-acetyl-L-cysteine (NAC; Sigma; 400 $mg.kg^{-1}.day^{-1}$; I.P.) daily prior to exposure to IH for 10 days. Rats exposed to normoxia receiving either saline or saline + NAC or saline + MnTMPyP served as controls.

Acute experiments were performed within ~16 h after terminating IH exposures in rats anesthetized with urethane (1.2 g/kg, Sigma; I.P.), and the surgical plane of anesthesia was assessed by the absence of limb withdrawal reflex in response to noxious pinching of toes.

Isolation of tissues

Whole brain tissues were removed from anesthetized rats, frozen in liquid nitrogen, and stored at −80°C until further analysis. In the experiments involving analysis of various brainstem regions, dorsal and ventral pons and dorsal and ventral medulla were dissected under ice-cold conditions.

Assay of TH activity

For the analysis of TH activity, either whole brainstem or dorsal and ventral medulla or dorsal and ventral pons were homogenized in 10 volumes of 10 m*M* potassium phosphate buffer, pH 7.4, containing complete protease inhibitor cocktail (EDTA-free; Roche, IN) and phosphatase inhibitor cocktail set I (Calbiochem, CA) at 4°C. The extract was centrifuged at 500 *g* for 10 min and the pellet containing cell debris and nuclei was discarded. The supernatant fraction was further centrifuged at 13,000 *g* for 30 min at 4°C and the resulting supernatant was passed through a Sephadex G-25 column to remove low molecular weight inhibitory molecules and immediately used for the assay of TH activity.

TH activity was determined by measuring the amount of L-DOPA formed from L-tyrosine, as described previously (27, 36, 37) with few modifications. Briefly, the reaction mixture (in a total volume of 250 μl) contained the following in *final* concentration: Tris-acetate, 100 m*M*, pH 5.8; 100 μM L-tyrosine; 400 μM (6R)-5,6,7,8-tetrahydrobiopterin dihydrochloride (Sigma) in 1 *M* 2-mercaptoethanol; 2500 units catalase; 50 μM 3-hydroxybenzyl hydrazine (NSD-1015, an inhibitor of L-aromatic amino acid decarboxylase); 400 μM $FeSO_4.7H_2O$, and tissue extract (200 ng protein equivalent). The concentration of BH_4 (400 μM) was selected based on preliminary studies comparing the effect of varying BH_4 concentration on TH activity in normoxic and IH brainstem tissues. To assess the specificity of the assay, control experiment was performed wherein L-tyrosine was replaced with D-tyrosine (100 μM). With D-tyrosine, DOPA formation was not detected in extracts derived from either normoxic or IH brainstem regions. After incubation of the reaction mixture at 37°C for 6 min, the reaction was terminated by the addition of 1 *M* perchloric acid containing 0.2 *M* EDTA at 4°C. A known amount of freshly prepared 3,4-dihydroxybenzylamine (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 using acid-activated aluminum oxide, and their levels were measured by HPLC combined with electrochemical detection as described below under "Analysis of DOPA". On average, ~78% DOPA was recovered during extraction, and all data were corrected for recovery losses during extraction and HPLC analysis and for variation in the amount of proteins. TH activity was expressed as nanomoles of DOPA formed from tyrosine per minute per milligram of protein. Protein concentration in tissue extracts was determined using DC protein assay kit from Bio-Rad using bovine serum albumin as the standard.

Analysis of DOPA

DOPA was analyzed by HPLC-ECD (Bioanalytical Systems) as described previously (27). Briefly, DOPA and DHBA (internal standard) were separated on a Prodigy ODS reverse

phase column (Phenomenex) by isocratic elution with a mobile phase consisting of 4% (vol/vol) acetonitrile, 0.1 *M* sodium nitrate, 0.08 *M* sodium dihydrogen phosphate, 200 mg/l sodium octyl sulfate, and 0.01% disodium EDTA adjusted to pH 2.7 with phosphoric acid. Under the experimental conditions, DOPA and DHBA were eluted at ~12 and 14 min, respectively, with an average recovery of ~78%. The chromatograms were recorded and analyzed using a Hitachi D-2500 Chromato-Integrator. The concentrations of DOPA and DHBA were determined using standard curves correlating their respective amounts to the integrated peak areas, corrected for recovery. The detection limit for DOPA was 50 picomoles.

Analysis of dopamine content

Brainstems from the control and IH rats were homogenized in 500 μl of 0.1 *N* $HClO_4$ containing 0.25 m*M* disodium EDTA, and the homogenates were centrifuged at 18,000 *g* for 15 min at 4°C. The clear supernatant was used for the analysis of catecholamines by HPLC-ECD as described previously (27).

Assay of protein phosphatase 2A (PP2A)

PP2A activity in the cytosolic and membrane fractions was assayed using EnzChek Phosphatase assay Kit (Invitrogen, OR) following the protocol provided by the manufacturer.

The dorsal and ventral medullae of the brainstem were homogenized in 25 m*M* Tris-HCl buffer, pH 7.4, containing 1 m*M* EDTA and complete protease inhibitor cocktail (Roche; homogenization buffer). Tissue extracts were centrifuged at 500 *g* for 15 min to remove the cell debris and nucleus and then at 16,000 *g* for 1 h at 4°C to separate cytosolic (supernatant) and membrane-enriched (pellet) fractions. Both the cytosolic and membrane-enriched fractions were stored at −80°C until further analysis. Prior to PP2A assay, the membrane fraction was thawed and extracted in 200 μl of the homogenization buffer containing 0.5% (vol/vol) Triton X-100, at 4°C overnight. The membrane extract was spun at 16,000 *g* for 30 min and the supernatant was used for PP2A assay.

PP2A activity was determined by measuring the amount of 6, 8-difluoro-7-hydroxy-4-methyl-coumarin formed from 6, 8-difluoro-4-methylumbelliferyl phosphate in a continuous fluorescence based assay in the presence and absence of 2 n*M* okadaic acid. The reaction mixture in a total volume of 200 μl contains Tris-Cl, 50 m*M*, pH 7.4; 6,8-difluoro-4-methylumbelliferyl phosphate, 50 μM and soluble or membrane fractions (10 μg protein). The formation of 6,8-difluoro-7-hydroxy-4-methyl-coumarin was continuously monitored for 10 min in a Shimadzu spectrofluorometer using excitation and emission wavelengths of 358 and 455 nm, respectively. The concentration of 6,8-difluoro-7-hydroxy-4-methyl-coumarin was determined from a standard curve correlating the concentration of the product to the corresponding fluorescence intensity values. PP2A activity is defined as the amount of okadaic acid (2 n*M*) inhibitable phosphatase activity and expressed as micromoles per minute per milligram protein.

Assay of protein kinases

For the assay of protein kinases, dorsal and ventral medullae from control and IH exposed rats were homogenized in 25 m*M* Tris-Cl, pH 7.4, containing complete protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail set I (Calbiochem) at 4°C. The homogenate was centrifuged at 13,000 *g* for 30 min at 4°C and the resulting clear supernatant was used for protein kinase assay.

Protein kinase A (PKA) activity was measured using commercially available PKA kinase activity assay kit (EKS-390A; Assay Designs, MI). Phosphorylation of PKA-specific synthetic peptide substrate was measured using a polyclonal antibody specific to a phosphorylated form of the substrate. Using a standard curve relating the amount of active PKA and the level of substrate phosphorylation, PKA activity was determined and expressed as nanogram of active PKA per mg of protein.

For the determination of Ca^{2+}/calmodulin-dependent kinase II (CaMKII) activity, a commercially available CycLex® CaMKII Assay Kit (CY-1173; MBL Corporation, MA) was used as per the manufacturers' instructions. To quantify CaMKII activity, a standard curve correlating the amount of active CaMKII and the level of phosphorylation of Syntide-2, a CaMKII specific substrate was constructed and enzyme activity was expressed as nanogram of active CaMKII per mg of protein.

To assay MAP kinase/extracellular signal-regulated kinase 1 and 2 (ERK1/2) activity, the level of phosphorylated ERK1/2 was determined by immunoblot analysis of tissue extracts as described in the following section. For immunolabeling of ERK1/2, antibodies specific to total and phosphorylated forms of ERK1/2 [phospho-p44/42 (Thr202/Tyr204)] were used (Cell Signaling Technology, MA; each at a dilution of 1:1,000).

Immunoblot analysis of TH and PP protein expressions

Equal amounts of protein (20 μg) from the control and IH tissue extracts were separated on 10% polyacrylamide slab gels containing sodium dodecyl sulfate and transferred to PVDF membrane. The transfer efficiency of proteins was assessed using brief Ponceau S staining. Nonspecific binding sites on the membrane were blocked by incubation with 5% milk in Tris-buffer containing saline and 0.1% (vol/vol) Tween-20 for 1 h. Blots were subsequently incubated with appropriate antibody for 18 h at 4°C. The following antibodies were used: anti-TH (Cell Signaling Technology; at 1:2,000 dilution); anti-phospho TH (Ser-19 and Ser-31, Chemicon International Inc, CA; each at 1:2,500 dilution); anti-phospho TH Ser-40 (Cell Signaling Technology; at 1: 2,500 dilution); anti-PP2A (Cell Signaling Technology; at 1:1,000 dilution); anti-PP2C (Epitomics, CA; at 1:1,000 dilution); anti-PP4 (Santa Cruz Biotech Inc., CA; at 1:1,000 dilution); anti-PP5 (BD Biosciences, CA; at 1:2,000 dilution); anti-PP6 (Exalpha Biologicals Inc., MA; at 1:1,000 dilution). After washing steps, the blots were incubated with horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse IgG (Santa Cruz Biotech Inc.; each at 1:10,000) for 1 h at 4°C. The immunoreactive total and phosphorylated forms of TH and various protein phosphatases were identified using ECL detection kit from Amersham Biosciences. The relative intensity of *major* immunoreactive protein band, as indicated by arrow in the figure legends was quantified using the Image J 1.37v software and the values were expressed as fold change with respect to normoxic control (*i.e.*, Normoxia = 1). As a loading control, the expression level of β-actin (42-kDa) was monitored.

In vitro dephosphorylation of TH by PP2A

Brainstem extracts from the control and IH rats were incubated in the presence of 0.2 units of the catalytic subunit of PP2A (Calbiochem) in 25 m*M* Tris-Cl buffer, pH 7.0, for 15 min at 25°C. For control samples, the extracts were incubated with buffer. Following dephosphorylation, the reaction mixture was assayed for TH activity as described above.

Assay of Thio-Barbituric Acid Reactive Substances (T-BARS)

The brainstem medullary regions were homogenized in 20 m*M* phosphate buffer, pH 7.4 and the homogenates were centrifuged in an Eppendorf microcentrifuge at 500 *g* for 10 min at 4°C. The level of lipid oxidation was determined as T-BARS, following the procedure as described (28, 44). Briefly, 100 μl of either sample or suitable concentrations of malondialdehyde (MDA) standard was added to 50 μl of SDS (8.1%), 375 μl of acetic acid (20%), and 375 μl of thiobarbituric acid (0.8%). The samples were incubated at 90°C for 60 min followed by incubation at 4°C for 10 min. The reaction mixture was centrifuged at 800 *g* for 15 min and the absorbance of the resulting supernatant was monitored at 532 nm. The level of T-BARS was expressed as nanomoles of MDA formed per mg protein.

Experimental protocols

Series 1. TH activity was determined in the whole brainstem of rats exposed to 10 days of either normoxia or IH_{15s} or IH_{90s} ($n = 7$ rats in each group). TH activity was measured in triplicate as described above.

Series 2. TH activity as well as the total as well as Ser-19, Ser-31, and Ser-40 phosphorylated forms of TH protein expression were analyzed in triplicate and duplicate, respectively, in the extracts of dorsal and ventral medullary regions of brainstem from rats exposed to 10 days of normoxia or IH_{15s} or IH_{90s} or daily treated with either MnTMPyP or NAC prior to IH_{15s} for 10 days ($n = 7$ rats in each group). TH activity in the dorsal and ventral pons of rats was also assayed in rats exposed to 10 days of either normoxia or IH_{15s}. The effect of *in vitro* PP2A treatment on TH activity was determined in the dorsal and ventral medullary extracts of rats exposed to 10 days of either normoxia or IH_{15s} ($n = 7$ rats in each group).

Series 3. PP2A activity and protein expression of PP2A, PP2C, PP4, PP5, and PP6 were assayed in triplicate and duplicate, respectively, in the dorsal and ventral medulla of rats exposed to 10 days of either normoxia or IH_{15s} or IH_{90s} or daily treated with MnTMPyP prior to IH_{15s} for 10 days ($n = 7$ rats in each group).

Series 4. Enzyme activities of PKA, CaMKII, and ERK1/2 were assayed in triplicate in the dorsal and ventral medulla of rats exposed to 10 days of either normoxia or IH_{15s} or IH_{90s} or daily treated with MnTMPyP prior to normoxia or IH_{15s} for 10 days ($n = 7$ rats in each group).

Series 5. Thio-barbituric acid reactive substances (T-BARS) level in the dorsal and ventral medullary brainstem regions was determined in rats exposed to 10 days of either normoxia or IH_{15s} or IH_{90s} or daily treated with MnTMPyP or NAC prior to normoxia or IH_{15s} for 10 days ($n = 7$ rats in each group).

Series 6. DA content in the whole brainstem was determined in triplicate in rats exposed to 10 days of normoxia or IH_{15s} or daily treated with either MnTMPyP or NAC prior to normoxia or IH_{15s} for 10 days ($n = 7$ rats in each group).

Data analysis

All enzyme activity measurements were made in triplicate whereas immunoblot analyses were made in duplicate. Data derived from 7 independent experiments ($n = 7$ rats in each group) in each one of the 6 series as outlined above were expressed as mean ± SE. Statistical significance was evaluated by unpaired *t*-test or one-way ANOVA for repeated measures. p Values < 0.05 were considered significant.

Results

Effect of IH on TH activity

In the normoxic, IH_{15s}, and IH_{90s} brainstem extracts, TH activity, as measured by the formation of DOPA, increased linearly as a function of reaction time (Fig. 1A). TH activity in the normoxic brainstem was 60.6 ± 8.3 nmol/min/mg protein. TH activity, following 10 days of IH_{15s}, increased by 5.6-fold compared to normoxic control (NOR, $p < 0.01$; Fig. 1B). The IH_{15s}-induced increase in TH activity was associated with a significant elevation in DA level in the brainstem ($p < 0.01$; Fig. 1C). By contrast, exposure of rats to 10 days of IH_{90s} did not significantly alter either TH activity or DA level in the brainstem ($p > 0.05$; Fig. 1B and C).

To assess regional variations, if any, in IH-evoked increase in TH activity, the effects of IH_{15s} on TH activity in the dorsal and ventral medulla as well as dorsal and ventral pons were determined. The basal TH activity in the dorsal medulla was 16.2 ± 4.3 nmol/min/mg protein and was comparable to the activity seen in the dorsal pons as well as ventral pons and medulla. IH_{15s} increased TH activity in the dorsal and ventral medulla by 9.8- and 7.1-fold, respectively, compared to normoxic control (NOR, $p < 0.01$; Fig. 1D), whereas TH activity in either dorsal or ventral pons was not significantly altered ($p > 0.05$; Fig. 1E).

Effect of IH on TH protein level

To determine whether IH_{15s}-induced increase in TH activity is due to increase in TH protein, dorsal and ventral medullary extracts from normoxic and IH exposed rats were resolved by SDS-PAGE and probed with anti-TH antibody. Immunoblot analysis showed that IH_{15s} had no significant effect on TH protein expression in the dorsal and ventral medullae ($p > 0.05$; Fig. 2A and B), suggesting that the increase in TH activity by IH_{15s} is not due to an increase in TH protein level.

IH_{15s} increases serine phosphorylation of TH

Because alterations in serine phosphorylation of TH have been shown to markedly affect TH activity (4, 11), the role of serine phosphorylation in IH_{15s}-induced increase in TH activity was investigated by immunoblot analysis using antibodies specific to Ser-19-, Ser-31-, and Ser-40 phosphorylated

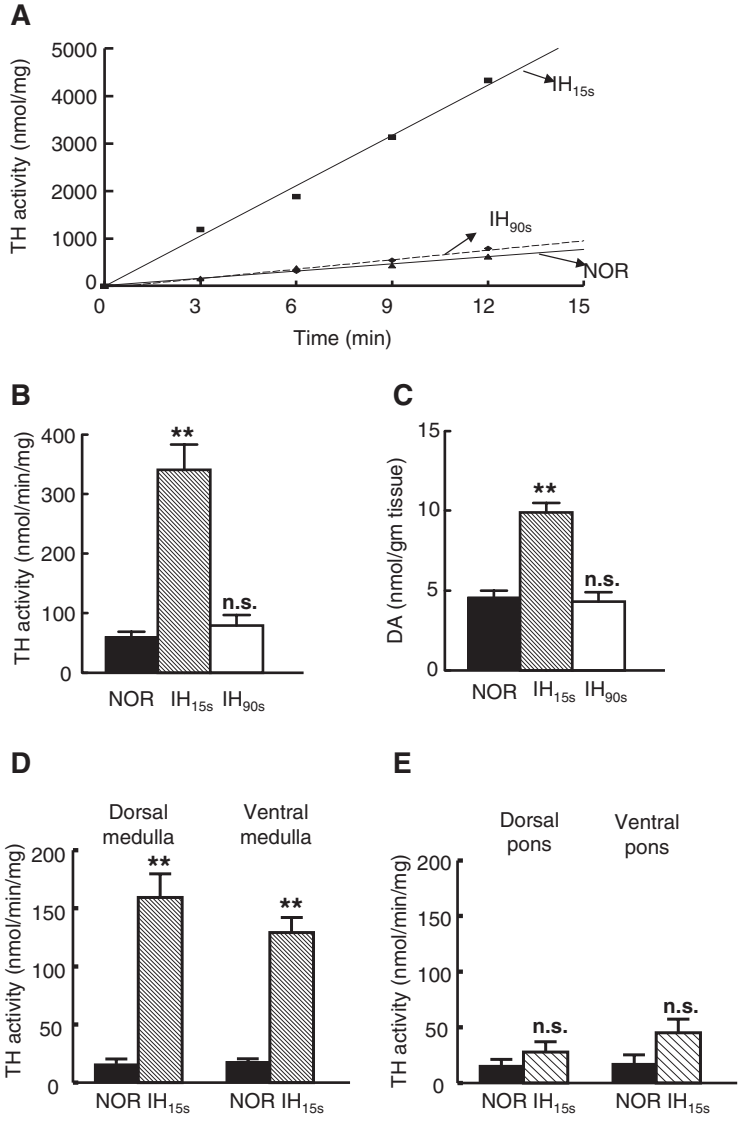

FIG. 1. Effects of two patterns of intermittent hypoxia (IH) on tyrosine hydroxylase (TH) activity and dopamine (DA) levels in the rat brainstem. Changes in TH activity as a function of reaction time are shown (**A**). TH activity was measured in the whole brainstem extracts of rats exposed to either IH_{15s} (alternating cycles of 15 s of 5% O_2 and 5 min of 21% O_2) or IH_{90s} (alternating cycles of 90 s of 10% O_2 and 90 s of 21% O_2) or normoxia (NOR) for 10 days, as described under "Methods". Effects of IH_{15s} and IH_{90s} on TH activity (**B**) and on DA levels (**C**) in the whole brainstem are shown. For DA analysis, brainstems were extracted with 0.1 *N* $HCLO_4$. DOPA formed during TH reaction and DA level in the whole brainstem were determined by HPLC combined with electrochemical detection as described in "Methods". TH activity is expressed as nanomoles of DOPA formed per min per mg protein, and DA as nanomoles per gram tissue. A comparison of the effect of IH_{15s} on TH activity in the dorsal and ventral medullary (**D**; *left panel*) and pontine (**E**; *right panel*) brainstem regions is shown. All measurements were made in triplicate and average data are shown as mean ± S.E. ($n = 7$ rats in each group). Asterisks denote $p < 0.01$. n.s., not significant ($p > 0.05$).

forms of TH. In the normoxic dorsal and ventral medulla, low levels of serine phosphorylated forms of TH were detected (Fig. 2A and B) and IH_{15s} evoked a robust increase in phosphorylation of TH at multiple serine residues. For instance, phosphorylation of TH at Ser-40 and Ser-31 was increased by 5.2- and 2.2-fold, respectively, in the IH dorsal medulla (Fig. 2A, left panels). On the other hand, IH_{15s} increased the level of phosphorylation of Ser-19, Ser-31, and Ser-40 of TH, albeit to different degrees in the ventral medulla. The rank order of IH-evoked increase in phosphorylation of TH was ser-19 < ser-40 < ser-31 (Fig. 2B; right panels). Taken together, the above findings suggest that IH markedly elevated phosphorylation of TH at Ser-31 and Ser-40 in the dorsal and ventral medulla with a concomitant robust increase in TH activity.

Evidence for the involvement of protein phosphatase 2A (PP2A) in IH_{15s}-induced activation of TH

The steady state level of serine phosphorylation of TH *in vivo* is regulated by the combined activities of protein kinases and protein phosphatases (see Ref. 11 for references). Either downregulation of TH dephosphorylation or upregulation of TH phosphorylation may contribute to increased serine phosphorylation of TH. In the following experiments, we tested the above possibilities.

Available evidence suggests that PP2A, a serine/threonine protein phosphatase that is sensitive to low concentration of okadaic acid (2 n*M*) plays a major role in TH dephosphorylation and hence TH activation in the rat adrenal medulla and brain (2, 20, 30). Therefore, we examined whether downregulation of PP2A contributes to IH_{15s}-evoked increase in TH activity in brainstem medullary regions.

Nearly 70% of PP2A activity was expressed in the cytosolic fractions isolated from the medullary brainstem regions. PP2A activity was relatively higher in the dorsal than in the ventral medulla of normoxic rats (NOR; Fig. 3A). IH_{15s} decreased PP2A activity by nearly 80% in both dorsal and ventral medulla ($p < 0.01$; Fig. 3A). The IH_{15s}-induced decrease in PP2A activity was, in part, due to reduced PP2A protein expression (38 kDa; $p < 0.01$; Fig. 3B, C, and D). On the other hand, PP2A activity in the membrane-enriched fraction was unaffected by IH_{15s} (data not shown).

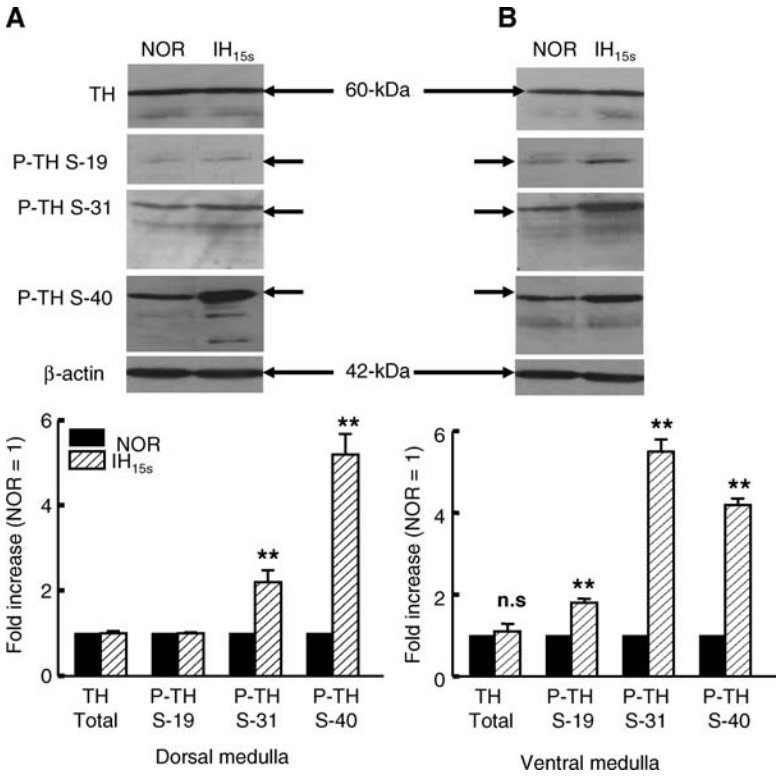

FIG. 2. Effects of IH_{15s} on total and serine phosphorylated forms of TH protein expression in the dorsal and ventral medullary brainstem regions. Equal amounts of proteins (20 μg) from normoxic and IH_{15s} extracts were used for immunoblot analysis. *Upper panels*: Representative examples of immunoblots of total and serine phosphorylated forms of TH (P-TH). *Bottom panels:* IH-induced fold change in total or serine phosphorylated forms of TH relative to normoxic control. Other experimental details are as described in "Methods". All measurements were made in duplicate and average data are shown as mean ± S.E. ($n = 7$ rats in each group). Asterisks denote $p < 0.01$; n.s., not significant ($p > 0.05$).

To assess whether IH_{15s} affect protein phosphatases other than PP2A that are sensitive to low concentration of okadaic acid (2 n*M*), we determined expression of PP4, PP5, and PP6, as well as PP2C which is insensitive to low concentration of okadaic acid in the extracts of normoxic and IH_{15s} brainstem medullary regions. Immunoblot analysis showed that IH_{15s} has no significant effect on the level of PP2C, PP4, PP5, and PP6 proteins ($p > 0.05$; Fig. 3B–D).

The results presented above suggest that IH_{15s} downregulates selectively PP2A in the medullary regions of the brainstem. To establish whether the downregulation of PP2A contributes to IH_{15s}-evoked TH activation, TH activity in the normoxic and IH_{15s} extracts of dorsal and ventral medulla was measured after incubating the extracts with the catalytic subunit of PP2A (0.2 units) for 15 min, and the results were compared with those derived from untreated controls. Pretreatment with the catalytic subunit of PP2A had no significant effect on TH activity in the normoxic controls, whereas it markedly reduced IH_{15s}-evoked increase in TH activity in the dorsal and ventral medulla (Fig. 4). Further increases in PP2A concentration had no additional effect on TH activity (data not shown).

IH_{15s} upregulates multiple protein kinases

In vitro and *in situ* studies showed that protein kinase A (PKA), Ca^{2+}/calmodulin-dependent kinase II (CaMKII), and MAP kinase/extracellular-signal regulated kinases 1 and 2 (ERK1/2) are the major protein kinases that phosphorylate TH at Ser-40, Ser-19, and Ser-31, respectively, (see ref. 11 for references). We determined whether IH_{15s} altered the activity of one or more of these protein kinases. Basal levels of active PKA, CaMKII, and ERK1/2 were comparable between dorsal and ventral medulla of brainstem (Fig. 5A–C). IH_{15s} upregulated active forms of PKA, CaMKII, and ERK1/2 in the dorsal and ventral medulla, albeit to different degrees. The rank order of increase in protein kinase activity by IH_{15s} was: ERK1/2 > PKA > CaMKII (Fig. 5A–D).

Effect of IH_{90s} on TH phosphorylation, protein phosphatase 2A, and protein kinases

As mentioned earlier, IH_{15s} but not IH_{90s}, evoked a robust increase in TH activity in brainstem medullary regions. The increase in TH activity by IH_{15s} was, in part, due to increased TH serine phosphorylation via mechanisms involving downregulation of protein phosphatase 2A and upregulation of protein kinases including PKA, CaMKII, and ERK1/2. We examined whether IH_{90s} can evoke a similar effect in dorsal and ventral medulla. In sharp contrast to IH_{15s}, neither TH phosphorylation (data not shown) nor PP2A activity nor levels of active PKA and CaMKII were affected by IH_{90s} (Table 1). Likewise, the level of phospho-ERK1/2 was also unaffected (data not shown).

Analysis of ROS generation by IH_{15s} and IH_{90s} and effects of antioxidants on IH_{15s}-induced functional alterations in TH

ROS generation is increased in central and peripheral tissues of rodents exposed to IH (28, 40, 44, 52). The following experiments were performed to determine whether IH_{15s} and IH_{90s} both facilitate ROS generation equally in the dorsal and ventral medulla and to assess whether ROS contributes to IH_{15s}-induced TH activation and the ensuing elevation in dopamine level, as well as the simultaneous upregulation of

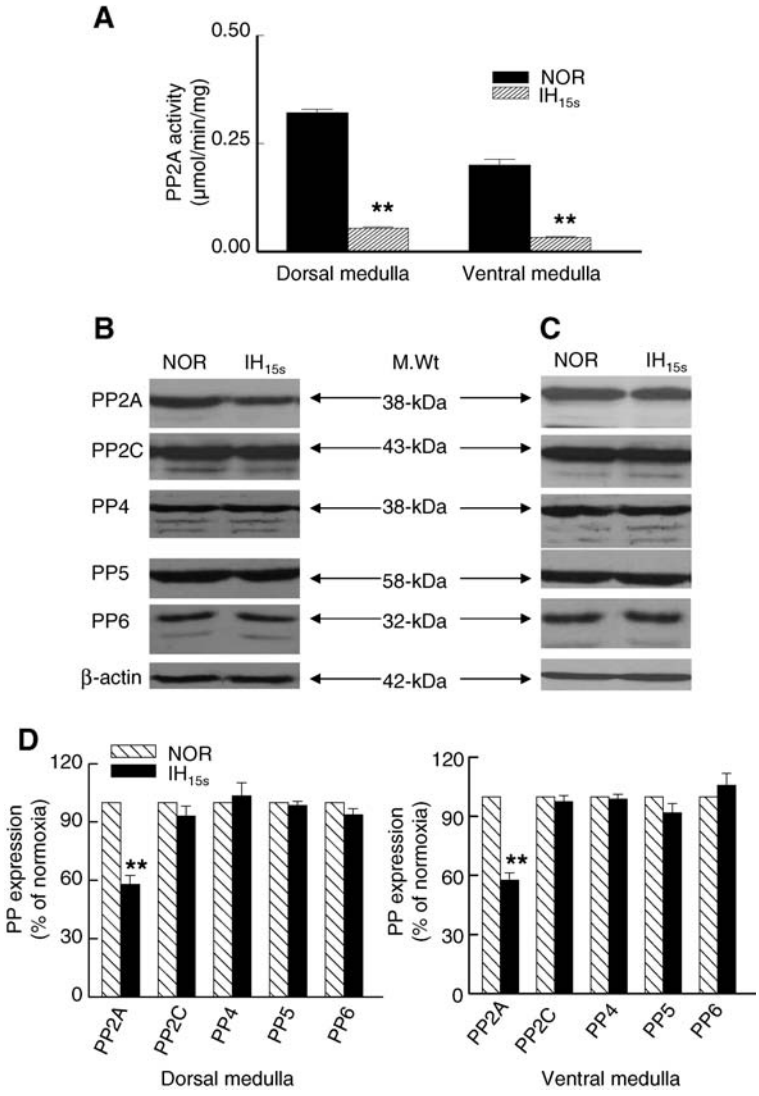

FIG. 3. Changes in PP2A enzyme activity and protein phosphatase (PP) expression in the dorsal and ventral medulla by IH_{15s}. Effect of IH on PP2A enzyme activity is shown (**A**). PP2A activity was determined by procedures as described under "Methods" and is expressed as micromoles per minute per mg protein. Representative immunoblots showing IH_{15s}-induced changes in PP expression in the dorsal (**B**) and ventral (**C**) medulla are presented. Equal amounts of proteins (20 μg) from normoxic (NOR) and IH_{15s} medullary extracts were used for immunoblot analysis. Changes in PP expression as percent of normoxia are shown in (**D**). All measurements were made in triplicate and average data are shown as mean $\pm$ S.E. ($n = 7$ rats in each group). Asterisks denote $p < 0.01$.

protein kinases and downregulation of PP2A and the resultant increase in serine phosphorylation of TH in the medullary brainstem regions.

An increase in the level of MDA was used as an index of ROS generation (28, 44). In the dorsal and ventral medulla, IH_{15s} increased MDA level by nearly 3.8-, and 2.5-fold, respectively compared to normoxic control (Fig. 6A). Although MDA level was elevated in brainstem medullary regions of rats exposed to IH_{90s}, the increase was much lower than that seen with IH_{15s} (Fig. 6B).

To determine whether antioxidants prevent IH_{15s}-, and IH_{90s}-induced increase in MDA level as well as IH_{15s}-evoked structural and functional changes in TH, rats were treated daily either with MnTMPyP, a membrane permeable $O_2^{\cdot -}$ scavenger or with NAC, an antioxidant and then exposed to IH or normoxia for 10 days. MnTMPyP and NAC treatments nearly abolished IH_{15s} and IH_{90s}-evoked elevation of MDA level (Fig. 6A and B). Notably, these antioxidants markedly attenuated IH_{15s}-induced increases in TH activity (Fig. 6C) and dopamine levels (Fig. 6D). However, neither MnTMPyP nor NAC affected TH activity and DA level in the normoxic controls (data not shown). More importantly, antioxidant reversed IH_{15s}-induced decrease in PP2A activity (IH_{15s} *versus* IH_{15s} + MnTMPyP; $p < 0.01$; Fig. 7A), as well as the increases in active PKA and CaMKII ($p < 0.01$; Fig. 7B and C) and in the level of phospho-ERK1/2 (data not shown) with a parallel attenuation of IH_{15s}-evoked increases in Ser-31 and Ser-40 phosphorylation of TH (IH_{15s} *versus* IH_{15s} + antioxidants; $p < 0.01$; Fig. 7D) in the dorsal and ventral medulla.

Discussion

A major finding of the present study is that IH, in a pattern-specific manner, increased TH activity in the rat brainstem medullary regions; IH_{15s} induced a robust increase in TH activity, whereas in sharp contrast, IH_{90s} had no significant effect. The increase in TH activity by IH_{15s} is consistent with a previously reported increase in TH activity in PC12 cells by an IH pattern similar to that used in the present study (27). The ineffectiveness of IH_{90s} to upregulate TH activity in brainstem medullary regions is in accord with the lack of stimulation of TH activity reported previously in the rat brainstem using a similar IH_{90s} protocol (17). The possible signaling mechanisms that may contribute to this contrasting

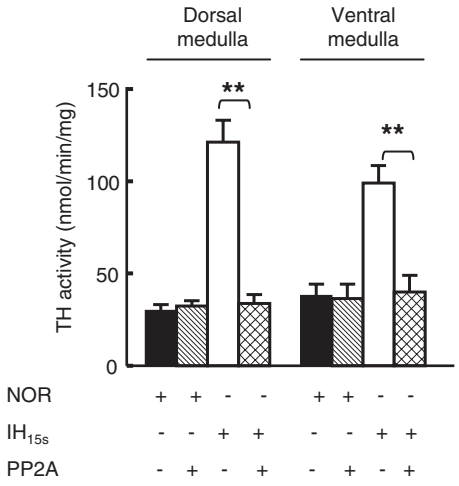

FIG. 4. Reversal of IH_{15s}-induced increase in TH activity by PP2A. TH activity was determined in the extracts of dorsal and ventral medulla derived from rats exposed to normoxia (NOR) or IH_{15s} for 10 days before and after dephosphorylation. For dephosphorylation, aliquots of the medullary extracts were treated with 0.2 units of the catalytic subunit of PP2A for 15 min, as described in the "Methods". All measurements were made in triplicate and average data are shown as mean ± S.E. ($n = 7$ rats in each group). Asterisks denote $p < 0.01$.

pattern-specific effect of IH on TH activity will be discussed in a later section.

Interestingly, IH_{15s} exerts a regio-specific effect on TH activity. Despite its expression in both pontine and medullary brainstem regions, TH activity was augmented by IH_{15s} only in the dorsal and ventral medulla. Since a similar increase in TH activity was not seen in pons, it is likely that the potentiating effect of IH_{15s} on TH activity is primarily restricted to the medullary brainstem regions. It is interesting to note that IH_{15s}, in sharp contrast to its effect on TH, uniformly increased the activity of peptidyl glycine α-amidating mono-oxygenase, an O_2 requiring, rate-limiting enzyme in the synthesis of C-terminal alpha amidated neuropeptides (*e.g.*, substance P) in both medulla and pons (45). The mechanism(s) that contributes to the differential responses of TH and PAM that are monooxygenases to IH_{15s} in the pontine brainstem region, however, remains to be determined.

IH_{15s}-induced increase in TH activity was accompanied by an increase in DA in the medullary brainstem regions. The IH_{15s}-induced elevation in DA may arise either from an increased synthesis or decreased degradation of DA. Since IH_{15s} also increased the level of dihydroxyphenylacetic acid, a major oxidative degradation product of DA (Kumar *et al.*, unpublished observation), it is likely that IH_{15s}-induced increase in DA in the brainstem is primarily due to increased TH-mediated synthesis.

How does IH_{15s} increase TH activity? TH activity is known to be regulated *in vivo* by several factors that include altered protein expression, serine phosphorylation, and feed-back product inhibition. Since the total TH protein expression per se remained the same between the normoxic and IH_{15s} medullae, the increase in TH activity by IH_{15s} is likely due to enzyme activation. Phosphorylation and/or dephosphorylation also contribute to long-term regulation of TH activity. Thus, increased phosphorylation at either Ser-31 or Ser-40, alone or in combination with Ser-19, augmented TH enzyme activity either by affecting the V_{max} or K_m (5, 50). Our results showed that TH phosphorylation at Ser-40 and Ser-31 is markedly upregulated, paralleling with the robust increase in TH activity seen in the medullary regions of IH_{15s} exposed rats. Taken together, the above findings suggest that increased serine phosphorylation could be a major mechanism by which IH_{15s} evokes TH activation in brainstem medullary regions.

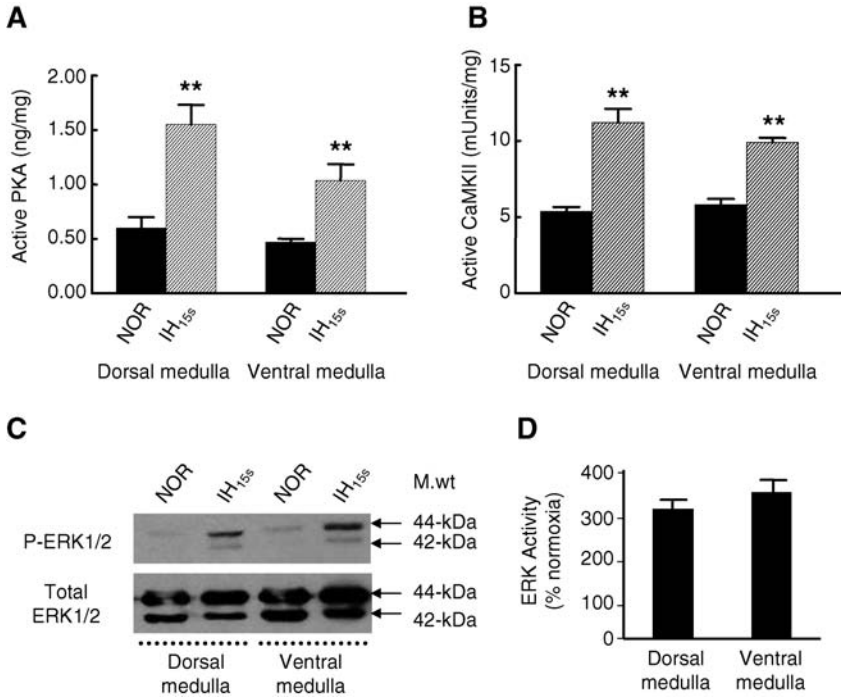

FIG. 5. Activation of protein kinases by IH_{15s}. Effects of IH_{15s} on the activity of protein kinase A (PKA; **A**), Ca^{2+}/calmodulin-dependent kinase II (CaMKII; **B**), and MAP kinase/extracellular signal regulated kinase 1 and 2 (ERK1/2; **C**) in the dorsal and ventral medulla are shown. Active PKA and CaMKII were determined using commercially available Kinase Assay Kit as per manufacturer's instructions. Activity of ERK1/2 was assessed by immunoblot analysis using antibodies specific for total- and phospho-ERK1/2 (P-ERK1/2). All measurements were made in triplicate and average data are shown as mean ± S.E. ($n = 7$ rats in each group). Asterisks denote $p < 0.01$.

Table 1. Effects of IH_{90s} on PP2A, PKA and CaMKII Activities in the Rat Dorsal and Ventral Medullary Brainstem Regions

	PP2A activity (μmol/min/mg)*		*PKA activity (ng of active PKA/mg)*		*CaMKII activity (ng of activeCaMKII/mg)*	
Gas challenge	*Dorsal medulla*	*Ventral medulla*	*Dorsal medulla*	*Ventral medulla*	*Dorsal medulla*	*Ventral medulla*
Control (NOR)	0.34 ± 0.08	0.22 ± 0.02	0.6 ± 0.2	0.4 ± 0.1	5.4 ± 0.3	5.8 ± 0.4
IH_{90s}	0.31 ± 0.11 (n.s.)	0.21 ± 0.04 (n.s.)	0.7 ± 0.3 (n.s.)	0.5 ± 0.4 (n.s.)	6.1 ± 0.6 (n.s.)	5.3 ± 0.5 (n.s.)

*Okadaic (2nM) acid inhibitable; All measurements were made in triplicate and average data are shown as mean ± S.E. ($n = 7$ rats in each group). n.s.-not significant, $p > 0.05$.

The steady-state level of TH phosphorylation *in vivo* is altered by protein kinases (via phosphorylation; 1, 4, 11, 15, 16, 29) and protein phosphatases (via dephosphorylation; 2, 3, 7, 14, 20, 30). Either an increase in the activity of protein kinases or downregulation of protein phosphatases would account for IH_{15s}-induced increase in serine phosphorylation of TH. Our results showed that IH_{15s} increased the levels of active forms of PKA, CaMKII, and ERK1/2, albeit to different degree, in the brainstem medullary regions. Since *in vitro,* as well as *in situ,* studies showed the involvement of PKA and CaMKII in Ser-40 phosphorylation (11) and ERK1/2 in Ser-31 phosphorylation (11, 22), it is likely that the increased activities of these protein kinases might have contributed to IH_{15s}-mediated robust phosphorylation of TH at Ser-40 and Ser-31. The finding that inhibitors specific to either PKA or CaMKII not only attenuated IH-evoked increase in TH phosphorylation but also TH activation in PC12 cells lends support for such a possibility (27). In addition to activation of protein kinases, IH_{15s} markedly decreased PP2A activity in the medullary brainstem regions which is, in part, due to a concomitant reduction in PP2A protein expression. Previous studies in the adrenal medulla and striatum showed that TH dephosphorylation is primarily mediated by okadaic acid-sensitive PP2A with a minor contribution from okadaic acid-insensitive PP2C (2, 3, 7, 20, 30). The findings that IH_{15s} had no significant effect on the expression of either okadaic acid-insensitive (PP2C) or other okadaic acid-sensitive protein phosphatases including PP4, PP5, and PP6, and that post-IH PP2A treatment reversed IH-induced increase in TH activity provide an indirect evidence for a critical role of PP2A in IH_{15s}-induced facilitation of TH phosphorylation. However, additional experiments involving reversal of IH_{15s}-evoked TH activation via overexpression of PP2A are needed to further confirm the modulatory role of PP2A in TH activation by IH. Furthermore, it is plausible that PP2A downregulation by IH_{15s} is functionally coupled to activation of protein kinases that are implicated in TH phosphorylation. Several studies support such a possibility. For instance, in cerebral cortex of

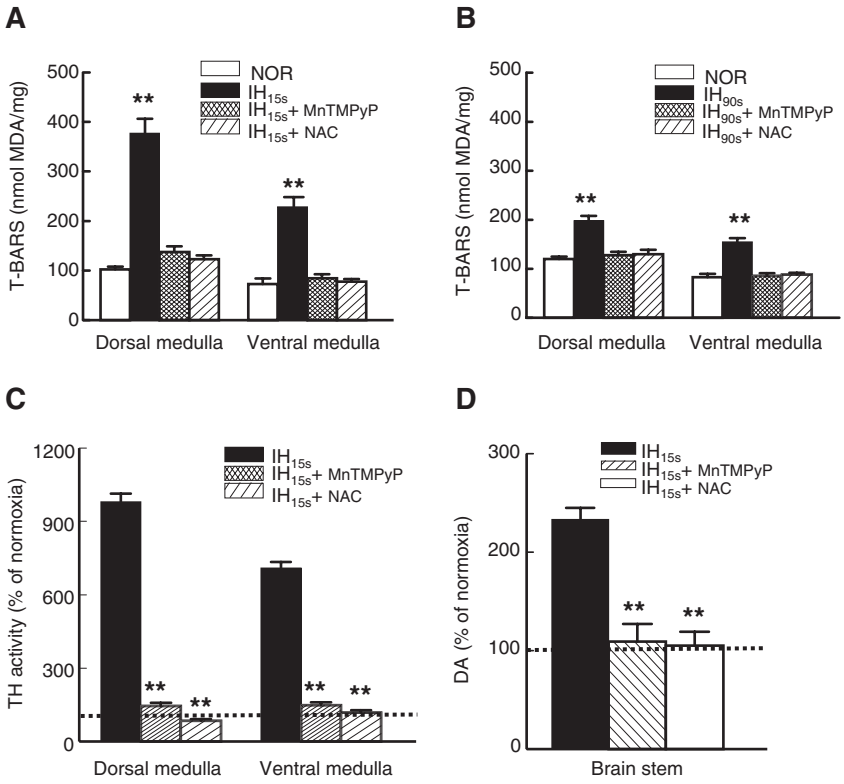

FIG. 6. IH-evoked ROS generation and effects of antioxidants on IH-induced ROS generation, increase in TH activity, and elevation in DA level in the dorsal and ventral medulla. The effects of IH_{15s} (**A**) and IH_{90s} (**B**) on T-BARS level in dorsal and ventral medullary homogenates are shown. MnTMPyP, a membrane permeable superoxide anion mimetic and *N*-acetyl-L-cysteine (NAC; a scavenger of reactive oxygen species) were administered (i.p.) daily prior to exposure of rats to either normoxia (NOR) or IH as described in "Methods". T-BARS level was expressed as nanomoles of malondialdehyde (MDA) formed per mg protein. Effects of MnTMPyP and NAC on IH_{15s}-induced increase in TH activity (**C**) and elevation of DA (**D**) are shown. DA was measured in whole brainstem extracts. All measurements were made in triplicate and average data are shown as mean ± S.E. ($n = 7$ rats in each group). Asterisks denote $p < 0.01$.

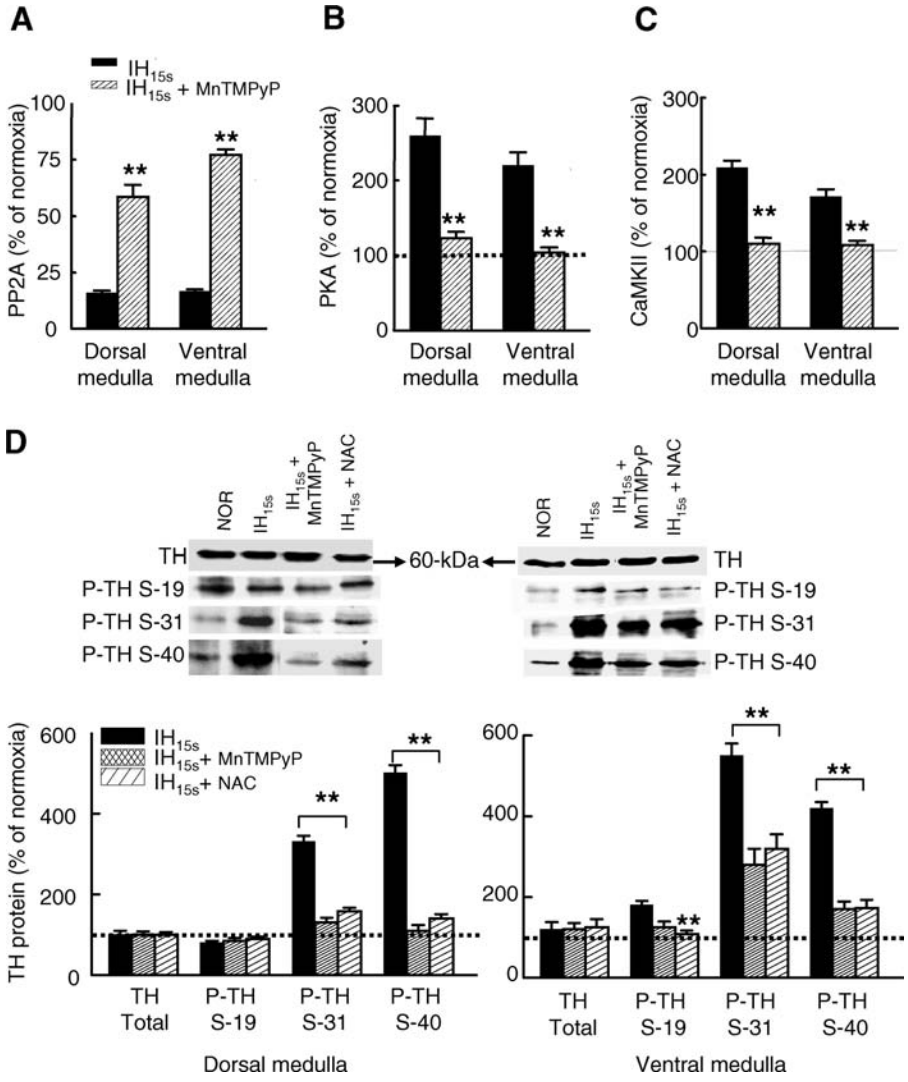

FIG. 7. Reversal of IH_{15s}-induced decrease in PP2A activity and the increase in PKA and CaMKII activity and serine phosphorylation of TH by antioxidants. The effects of MnTMPyP on IH_{15s}-evoked decrease in PP2A activity (**A**) and increases in PKA (**B**), CaMKII (**C**), and phosphorylation of TH at S-19, S-31 and S-40 (P-TH; **D**) in the dorsal and ventral medullary brainstem regions are presented. Other experimental details are as described in the legends of Figs. 3 and 5. The data are expressed as percent of normoxic control. All measurements were made in triplicate and average data are shown as mean ± S.E. ($n = 7$ rats in each group). Asterisks denote $p < 0.01$.

newborn piglets, hypoxia-evoked increase in CaMK IV activity, a modulator of programmed cell death, has been attributed to decrease in PP2A activity (48). Likewise, survival of macrophages during hyperoxia has been shown to involve sustained activation of ERK which is mediated by PP2A downregulation (39). Thus, IH_{15s}-evoked activation of a subset of protein kinases via PP2A downregulation may provide a positive feed forward mechanism for sustained TH phosphorylation, a possibility which needs to be further investigated. Taken together, our results suggest that IH_{15s} via tilting the balance between protein kinases and protein phosphatases facilitates robust TH phosphorylation.

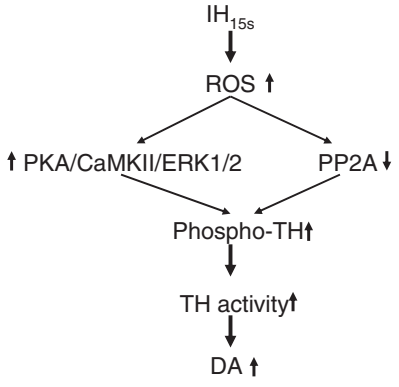

FIG. 8. Schematic presentation of ROS-dependent downregulation of PP2A and upregulation of protein kinases in IH_{15s}-induced activation of TH.

ROS signaling plays a critical role in eliciting systemic and cellular responses to IH (28, 40, 47, 49). The following lines of evidence suggest that ROS is an upstream signaling molecule that plays a central role in IH-evoked TH activation and the resulting increase in DA levels in the rat medullary brainstem regions. First, IH increased ROS in the rat dorsal and ventral medulla as evidenced by increases in MDA level, a marker of increased ROS generation. Second, pre-treatment with two mechanistically distinct antioxidants, viz., MnTMPyP, a SOD mimetic and N-NAC, a precursor of glutathione, not only attenuated IH-induced increases in MDA level, TH activity, DA level, and activities of PKA, CaMKII, and ERK1/2, but also the elevation of serine phosphorylation of TH at Ser-31 and Ser-40 by IH. Interestingly, antioxidant also reversed IH-evoked downregulation of PP2A activity, suggesting a direct role for ROS in post-translational redox regulation of PP2A. Supporting such a possibility are the findings that exposure of

PC12 and SH-SY5Y cells to cadmium not only increases ROS but also inhibits PP2A and PP5 and that NAC reverses cadmium-induced PP2A inhibition (8). Taken together, the above observations suggest that ROS-mediated downregulation of PP2A contributes to IH_{15s}-induced post-translational serine phosphorylation and subsequent activation of TH. However, the cellular sources of ROS generation (cytosolic oxidases and complexes of mitochondrial electron transport chain) during IH_{15s} and the chemical identity of the ROS species ($O_2^{\cdot -}$ or H_2O_2 or OH^-) that mediate IH-induced functional alteration in TH in brainstem medullary regions, however, remain to be established. Recent studies in rat carotid body (41) and PC12 cells (51) suggest that NADPH oxidase-derived ROS may contribute to IH_{15s}-mediated responses. Furthermore, *in vitro* studies have shown that TH, under certain reaction conditions, facilitates H_2O_2 generation (21). Future studies, however, are required to determine whether ROS derived from NADPH oxidase and/or TH catalyzed reaction mediate IH_{15s}-evoked TH activation in the brainstem medullary regions.

It is noteworthy that IH_{15s}, but not IH_{90s}, augments TH activity. Since molecular oxygen is required for the catalytic activity of TH and hypoxia lowers tissue oxygen level, it is rather intriguing that IH with only short (15 s) but not long (90 s) duration of hypoxia facilitates TH activity. It is likely that the signaling mechanism(s) that are necessary for TH activation is fully developed in IH_{15s} whereas it is not sufficiently developed in IH_{90s}. Supporting such a possibility are the findings that the level of ROS which is critical for IH-induced activation of TH, is three times higher in brainstem medullary regions of rats exposed to IH_{15s} than those exposed to IH_{90s}. Moreover, ROS signaling mediated upregulation of PKA/CaMKII and downregulation of PP2A were entirely absent in IH_{90s} brainstem regions. Thus, IH_{15s} was able to induce a robust ROS generation reaching a threshold level necessary for the activation of ROS signaling pathway involving protein kinases and phosphatases for marked elevation in TH activity. The possibility remains that with IH_{90s} such robust ROS generation may be elicited after prolonged exposure to IH lasting several weeks. Thus, it is likely that for a given duration and type of tissue, the magnitude of ROS generation may vary among different IH patterns. The finding that mice exposed to IH consisting of alternating cycles of 4.9% O_2 for 30 s and 20.9% O_2 for 30 s for 1 or 4 weeks exhibited oxidative stress only in liver but not in the aorta and heart (24) supports such a possibility. Furthermore, IH_{90s}, unlike in the brainstem, was able to increase TH phosphorylation and catecholamines only in the carotid body but not in the adrenal medulla or superior cervical ganglion (23), suggesting that IH_{90s}-evoked responses are tissue specific. In marked contrast, with IH_{15s}, we observed significant elevations in adrenal medullary catecholamines (28) and robust sustained increase in TH activity in the adrenal medulla as well as in the superior cervical ganglion similar to those seen in brainstem medullary regions (Kumar *et al.*, unpublished observation). These observations suggest that IH_{15s} evokes a robust global TH activation in catecholamine expressing tissues.

In summary, our results provide evidence that IH_{15s}-induced increase in ROS initiates a cascade of reactions involving downregulation of PP2A and upregulation of protein kinases, resulting in sustained increase in phosphorylation at multiple serine residues and the ensuing activation of TH and increase in DA synthesis (Fig. 8). Interestingly, the increase in TH phosphorylation by IH_{15s} occurs in the absence of significant alteration in the overall TH protein expression and therefore constitutes as one of the major post-translational mechanisms activated by IH_{15s} for the regulation of enzymes associated with neurotransmitter synthesis. The finding that IH_{15s}-mediated activation of TH is coupled to increased DA level in the medullary brainstem regions suggests that IH-evoked changes in DA may, in part, contribute to cardiorespiratory abnormalities associated with recurrent apneas.

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Abbreviations

CaMK, calcium/calmodulin-dependent kinase; DA, dopamine; DHBA, 3,4-dihydroxybenzylamine; DOPA, dihydroxyphenylalanine; ERK, extracellular signal-regulated kinase; IH, intermittent hypoxia; MDA, malondialdehyde; MnTM-PyP, manganese (III) tetrakis (1-methyl-4-pyridyl) porphyrin pentachloride; NAC, *N*-acetyl-L-cysteine; NOR, normoxia; PC12, pheochromocytoma 12; PKA, protein kinase A; PP, protein phosphatase; ROS, reactive oxygen species; T-BARS, thio-barbituric acid reactive substances; TH, tyrosine hydroxylase.

Author Disclosure Statement

The authors have no commercial associations that might create a conflict of interest in connection with the submission of the manuscript.

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