Neonatal Intermittent Hypoxia Leads to Long-Lasting Facilitation of Acute Hypoxia-Evoked Catecholamine Secretion From Rat Chromaffin Cells

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INTRODUCTION

Catecholamine (CA) secretion from the adrenal medulla is critical for maintaining homeostasis under a variety of stress conditions including hypoxia (Lagercrantz and Bistoletti 1977; Seidler and Slotkin 1985). In adult animals, hypoxia-evoked CA secretion from chromaffin cells is neurogenic and requires the activation of the sympathetic nervous system (Seidler and Slotkin 1985). In neonates, the sympathetic nervous system is not well developed (Seidler and Slotkin 1985). In this case, hypoxia still evokes CA secretion from neonatal chromaffin cells by directly affecting their excitability and elevating intracellular calcium ion concentration ([Ca\(^{2+}\)]\(_i\)).

The animals were unrestrained, freely mobile, and fed without restriction. The chamber was flushed with alternating cycles of nitrogen gas and room air. Inspired O\(_2\) levels reached a nadir of 5% O\(_2\) during hypoxia. O\(_2\) and CO\(_2\) levels in the chamber were continuously monitored and ambient CO\(_2\) levels were maintained between 0.2 and 0.5%. Control experiments were performed on age-matched rat pups exposed to normoxia. In the protocols involving antioxidant treatment, rat pups were given manganese (III) tetrakis(1-methyl-4-pyridyl) porphyrin pentachloride (MnTMPyP; Alexis Biochemicals, San Diego, CA; 5 mg·kg\(^{-1}\)·day\(^{-1}\), administered intraperitoneally [ip]), a membrane-permeable superoxide dismutase mimetic or N-acetyl cysteine (NAC; 800 mg·kg\(^{-1}\)·day\(^{-1}\), ip), every day prior to placing rats in the IH chamber. Rat pups treated with vehicle (saline) served as controls. To determine the effects of SH, rat pups along with their mother were exposed to hypobaric hypoxia (0.4 atm) for either 24 h or 5 days as described previously (Kumar et al. 2006). Acute experiments were performed on anesthetized pups (urethane 1.2 g·kg\(^{-1}\), ip) 6–10 h following either IH, SH, or normoxia.

METHODS

Experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Chicago. Experiments were performed on neonatal Sprague–Dawley rats pups (P0–P35).

Exposure to IH and SH

Rat pups (P0) along with their mothers were exposed to IH (15-s hypoxia/5-min normoxia; 8 h/day) or SH (hypobaric hypoxia, 0.4 atm) or normoxia (controls) from P0 to P5. IH treatment facilitated hypoxia-evoked CA secretion and elevations in [Ca\(^{2+}\)]\(_i\), CA, and ROS levels persisted in rats reared under normoxia for >30 days. In striking contrast, chromaffin cells from SH-treated animals exhibited attenuated hypoxia-evoked CA secretion. In SH-treated cells hypoxia-evoked elevations in [Ca\(^{2+}\)]\(_i\), NE and E contents, and ROS levels were comparable with controls. These observations demonstrate that: 1) neonatal IH and SH evoke opposite effects on hypoxia-evoked CA secretion from chromaffin cells, 2) ROS signaling mediates the facilitatory effects of IH, and 3) the effects of neonatal IH on chromaffin cells persist into adult life.

SOUVANNAKITTI D, Kumar GK, Fox A, Prabhakar NR. Neonatal intermittent hypoxia leads to long-lasting facilitation of acute hypoxia-evoked catecholamine secretion from rat chromaffin cells. J Neurophysiol 101: 2837–2846, 2009. First published April 1, 2009; doi:10.1152/jn.00036.2009. The objective of the present study was to examine the effects of intermittent hypoxia (IH) and sustained hypoxia (SH) on hypoxia-evoked catecholamine (CA) secretion from chromaffin cells in neonatal rats and assess the underlying mechanism(s). Experiments were performed on rat pups exposed to either IH (15-s hypoxia/5-min normoxia; 8 h/day) or SH (hypobaric hypoxia, 0.4 atm) or normoxia (controls) from P0 to P5. IH treatment facilitated hypoxia-evoked CA secretion and elevations in the intracellular calcium ion concentration ([Ca\(^{2+}\)]\(_i\)), and these responses were attenuated, but not abolished, by treatments designed to eliminate Ca\(^{2+}\) flux into cells (Ca\(^{2+}\)-free medium or Cd\(^{2+}\)), indicating that intracellular Ca\(^{2+}\) stores were augmented by IH. Norepinephrine (NE) and epinephrine (E) levels of adrenal medullae were elevated in IH-treated pups. IH treatment increased reactive oxygen species (ROS) production in adrenal medullae and antioxidant treatment prevented IH-induced facilitation of CA secretion, elevations in [Ca\(^{2+}\)]\(_i\), by hypoxia, and the up-regulation of NE and E. The effects of neonatal IH treatment on hypoxia-induced CA secretion and elevation in [Ca\(^{2+}\)]\(_i\), CA, and ROS levels persisted in rats reared under normoxia for >30 days. In striking contrast, chromaffin cells from SH-treated animals exhibited attenuated hypoxia-evoked CA secretion. In SH-treated cells hypoxia-evoked elevations in [Ca\(^{2+}\)]\(_i\), NE and E contents, and ROS levels were comparable with controls. These observations demonstrate that: 1) neonatal IH and SH evoke opposite effects on hypoxia-evoked CA secretion from chromaffin cells, 2) ROS signaling mediates the facilitatory effects of IH, and 3) the effects of neonatal IH on chromaffin cells persist into adult life.

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Preparation of chromaffin cells and cell culture

Adrenal glands were harvested from IH, SH, and control rat pups anesthetized with urethane (1.2 g·kg⁻¹, ip). The adrenal cortex was removed and the medulla was cut into small pieces. Chromaffin cells were enzymatically dissociated using a mixture of collagenase P (2 mg/ml; Roche), DNase (25 µg/ml; Sigma), and bovine serum albumin (3 mg/ml; Sigma) at 37°C for 30 min, followed by a 15-min digestion in 0.03% trypsin/EDTA (Invitrogen) and DNase 50 µg/ml (Sigma). Cells were centrifuged at 200 g for 15 min at 4°C and were plated onto collagen-coated coverslips (type VII; Sigma) and maintained at 37°C in a 5% CO₂ incubator for 12–24 h. The growth medium consisted of F-12 K medium (Invitrogen) supplemented with 10% horse serum, 5% fetal bovine serum, and 1% penicillin/streptomycin/glutamine cocktail (Invitrogen).

Amperometry

Catecholamine secretion from chromaffin cells was monitored by amperometry using carbon-fiber electrodes as described previously (Grabner et al. 2006). The electrode was held at +700 mV versus a ground electrode using an NPI VA-10 amplifier to oxidize catecholamine transmitter. The amperometric signal was low-pass filtered at 2 kHz (eight-pole Bessel; Warner Instruments, Hamden, CT) and sampled into a computer at 10 kHz using a 16-bit A/D converter (National Instruments, Austin, TX). Records with root-mean-square (RMS) noise >2 pA were not analyzed. Amperometric spike features, quantal size, and kinetic parameters were analyzed using a series of macros written in Igor Pro (WaveMetrics) kindly supplied by Dr. Eugene Mosharov. The detection threshold for an event was set at four to five times the RMS noise and the spikes were automatically detected. The area under individual amperometric spikes is equal to the charge (pC) per release event, referred to as Q. The number of oxidized neurotransmitter molecules (N) was calculated using the Faraday equation, N = Q/ne, with n = 2 electrons per oxidized molecule of transmitter and where e is the elemental charge (1.603 × 10⁻¹⁹ coulombs). Because the number of events varied considerably from cell to cell, the data from each cell were averaged to provide a single number for the overall statistic using the technique described by Colliver et al. (2000).

Recording solutions and stimulation protocols

Amperometric recordings were made from adherent cells that were under constant perfusion (flow rate of about 1.0 ml/min: chamber volume = 80 µl). All experiments were performed at...
ambient temperature (23 ± 2°C), and the solutions had the following composition (in mM): 1.26 CaCl₂, 0.49 MgCl₂·6H₂O, 0.4 MgSO₄·7H₂O, 5.33 KCl, 0.441 KH₂PO₄, 137.93 NaCl, 0.34 Na₂HPO₄·7H₂O, 5.56 dextrose, and 20 Hepes (pH 7.35 and 300 mOsm). Normoxic solutions were equilibrated with room air (PO₂ = 146 mmHg). For challenging with hypoxia, solutions were degassed and equilibrated with appropriate gas mixtures that resulted in final medium PO₂ values of 30, 60, and 100 mmHg as

FIG. 2. Effects of IH on K⁺-evoked CA secretion from neonatal chromaffin cells. A: examples of K⁺-evoked CA secretion from chromaffin cell derived from P5 rats reared under normoxia (Control) or IH. The horizontal black bars represent the duration of K⁺ (40 mM KCl) challenge. B and C: average data of the effects of K⁺. B: the number of secretory events/min. C: average CA molecules released/event. Data shown are means ± SE. Control = 16; IH = 20 cells obtained from 3 different litters in each group. ***P < 0.0001.

FIG. 3. Effects of IH on hypoxia-induced intracellular calcium ion concentration ([Ca²⁺]ᵢ) changes in neonatal chromaffin cells. A: mean [Ca²⁺]ᵢ responses to hypoxia (PO₂ = 30 mmHg) determined every 2 s in individual chromaffin cells from P5 rats reared in normoxia (Control) or IH. Horizontal black bars represent the duration of the hypoxic challenge. B and C: average data of basal (B) and hypoxia-evoked [Ca²⁺]ᵢ changes (C) in chromaffin cells from control and IH-treated rat pups. Data presented are means ± SE from 33 and 38 cells in Control and IH, respectively. The cells were obtained from 3 different litters in each group. ***P < 0.0001.
Ca\textsuperscript{2+} was measured by blood gas analyzer. Ca\textsuperscript{2+}-free solutions contained 0.5 mM EGTA.

**Measurements of \([Ca^{2+}]_i\)**

\([Ca^{2+}]_i\) was monitored in chromaffin cells as described previously (Xie et al. 2004). Briefly, chromaffin cells were incubated in Hanks’ balanced salt solution (HBSS) with 2 \(\mu\)M fura-2 AM and 1 mg/ml albumin for 30 min and washed in a fura-2-free solution for 30 min at 37°C. The coverslip was transferred to an experimental chamber for recording. Background fluorescence at 340- and 380-nm wavelengths were obtained using an area of the coverslip devoid of cells. Data were continuously collected throughout the experiment. On each coverslip, four to eight chromaffin cells were selected and individually imaged. Image pairs (one at 340 and the other at 380 nm) were obtained every 2 s by averaging 16 frames at each wavelength. Background fluorescence was subtracted from the individual wavelengths and the 340-nm image was divided by the 380-nm image to provide a ratiometric image. Ratios were converted to free \([Ca^{2+}]_i\) by comparing data to fura-2 calibration curves made in vitro by adding fura-2 (50 \(\mu\)M free acid) to solutions that contained known concentrations of calcium (0–2,000 nM). The recording chamber was continually perfused with fresh solution from gravity-fed reservoirs.

**Measurement of catecholamine content**

Experiments were performed on freshly harvested adrenal medullae from anesthetized rats. Catecholamines were extracted with 0.1 N HClO\textsubscript{4} containing 10 mM EDTA-Na\textsubscript{2} and assayed by high pressure liquid chromatography (HPLC) coupled with electrochemical detection (HPLC-ECD) as previously described (Kumar et al. 2006). Norepinephrine and epinephrine contents were expressed as nanomoles per milligram of protein.

**Measurement of malondialdehyde (MDA)**

Adrenal medullary tissue was homogenized in 10 volumes of 20 mM phosphate buffer (pH 7.4) at 4°C and centrifuged at 500 g for 10 min at 4°C. MDA levels were analyzed in supernatants as previously described (Peng et al. 2006) and expressed as nanomoles of MDA formed per milligram of protein.
from control pups (control IH-treated pups responded to hypoxia than cells derived from chromaffin cells in the hypoxic stimulus, CA secretion by a depolarizing stimulus was determined as an index of ROS levels from adrenal medullae from rat pups reared under normoxia (Control) or IH treated with antioxidant manganese (III) tetrakis(1-methyl-4-pyridyl) porphyrin pentachloride (MnTMPyP, 5 mg·kg⁻¹·day⁻¹; administered intraperitoneally from P0–P5 denoted by +) or vehicle (denoted by −). Data presented are means ± SE from 6 rat pups derived from 3 different litters in each group. **P < 0.001; n.s., not significant (P > 0.05). B: representative examples of hypoxia-induced CA secretion (PO₂ = 30 mmHg; at horizontal black bars) in control, IH, and IH + MnTMPyP-treated chromaffin cells. C and D: average data on the effects of graded hypoxia. C: the number of secretory events/min. D: the average CA molecules released per event. PO₂ = partial pressure of O₂ in mmHg. Data shown are means ± SE. Control = 17; IH = 20; IH + MnTMPyP = 14 cells from 3 different litters in each group. *P < 0.01; **P < 0.001; n.s., not significant (P > 0.05).

**FIG. 5.** Reactive oxygen species (ROS) mediate IH-evoked facilitation of hypoxia-induced CA secretion. A: malondialdehyde (MDA) levels were determined as an index of ROS levels from adrenal medullae from rat pups reared under normoxia (Control) or IH treated with antioxidant manganese (III) tetrakis(1-methyl-4-pyridyl) porphyrin pentachloride (MnTMPyP, 5 mg·kg⁻¹·day⁻¹; administered intraperitoneally from P0–P5 denoted by +) or vehicle (denoted by −). Data presented are means ± SE from 6 rat pups derived from 3 different litters in each group. **P < 0.001; n.s., not significant (P > 0.05). B: representative examples of hypoxia-induced CA secretion (PO₂ = 30 mmHg; at horizontal black bars) in control, IH, and IH + MnTMPyP-treated chromaffin cells. C and D: average data on the effects of graded hypoxia. C: the number of secretory events/min. D: the average CA molecules released per event. PO₂ = partial pressure of O₂ in mmHg. Data shown are means ± SE. Control = 17; IH = 20; IH + MnTMPyP = 14 cells from 3 different litters in each group. *P < 0.01; **P < 0.001; n.s., not significant (P > 0.05).

**RESULTS**

IH facilitates hypoxia-evoked CA secretion

Acute hypoxia-evoked robust CA secretion from control and IH-treated chromaffin cells and the secretory response was greater in IH-treated cells than that in controls (Fig. 1, A and B). Averaging the data from many cells showed that the number of secretory events as well as the amount of CA released per event evoked by moderate (PO₂ = 60 mmHg) and severe (PO₂ = 30 mmHg) hypoxia were significantly higher in IH compared with control cells (P < 0.001; Fig. 1, C and D). Furthermore, a larger percentage of cells from IH-treated pups responded to hypoxia than cells derived from control pups (control = 17 of 23 cells = 74% vs. IH = 20 of 22 cells = 90%).

To determine whether the effects of IH were selective to the hypoxic stimulus, CA secretion by a depolarizing stimulus (40 mM K+ −) was determined. CA secretion evoked by K+− was significantly greater in IH than from control cells, arising from increases both in the number of secretory events and in the amount of CA released per secretory event (P < 0.0001; Fig. 2).

The above-cited results showed that IH increases the amount of CA released per secretory event, which could be due to an increase in catecholamine content or number of vesicles undergoing fusion before exocytosis (i.e., compound exocytosis). To test the latter possibility, CA content of adrenal medullae was determined from control and IH-treated rat pups. Norepinephrine and epinephrine contents were significantly elevated in IH-treated adrenal medullae compared with controls (norepinephrine content: Control = 19.3 ± 1.4 vs. IH 49 ± 3.7 nmol/mg protein; epinephrine content: Control = 170 ± 14 vs. IH = 373 ± 11 nmol/mg protein; P < 0.001; n = 6 pups each).

Effects of IH on hypoxia-evoked [Ca²⁺], in chromaffin cells

In neonatal chromaffin cells, hypoxia-evoked CA secretion requires elevation of [Ca²⁺], (Inoue et al. 1998; Takeuchi et al. 2001). Comparison of [Ca²⁺], responses revealed both the baseline [Ca²⁺], and the magnitude of hypoxia-induced elevation in [Ca²⁺], were significantly higher in IH-treated cells than in the controls (P < 0.0001; Fig. 3).

To assess the contribution of extracellular Ca²⁺ flux, hypoxia-evoked CA secretion was monitored in the presence of Ca²⁺-free medium (i.e., medium with zero calcium and 0.5 mM of EGTA). As shown in Fig. 4, in control cells hypoxia-evoked CA secretion was suppressed in Ca²⁺-free medium, whereas small but significant CA secretion could still be elicited in IH-treated cells (Fig. 4, A and B). Similar results were also obtained with medium containing 300 µM Cd²⁺, a broad spectrum blocker of voltage-gated Ca²⁺ channels (Fig. 4B). The residual CA secretion in the absence of Ca²⁺ was significantly increased in IH-treated cells compared with controls (P < 0.01; Fig. 4C).

**Data analysis**

Statistical analyses between experimental groups are presented as means ± SE and Student’s t-test was used for statistical comparisons between two groups. P values < 0.05 were considered significant.

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of Ca\(^{2+}\) flux seen in IH-treated cells might be due to the mobilization of intracellular Ca\(^{2+}\). To assess this possibility, we examined the effects of 10 mM caffeine, which mobilizes Ca\(^{2+}\) stores from sarcoplasmic reticulum, on [Ca\(^{2+}\)]i, in control and IH-treated cells. The elevations in [Ca\(^{2+}\)]i by caffeine were slightly but significantly higher in IH compared with control cells (Fig. 4C; Control +380 ± 8 vs. IH = +426 ± 7 nM; an increase of ~46 nM in IH cells; Control vs. IH; P < 0.05; n = 9 and 11 cells for Control and IH, respectively).

**ROS signaling mediates chromaffin cell responses to IH**

Previous studies have shown that reactive oxygen species (ROS) mediate cellular responses to IH (Prabhakar et al. 2007). To determine whether ROS do play a role in chromaffin cell responses to IH, malondialdehyde (MDA) levels in adrenal medullae were monitored as an index of ROS generation (Peng et al. 2006; Ramanathan et al. 2005). The MDA levels were elevated by 60% in adrenal medullae from IH compared with control pups (P < 0.001; Fig. 5A) and this response was prevented by systemic administration of MnTMPyP, a cell-permeable antioxidant.

To determine whether ROS contribute to chromaffin cell responses to IH, rat pups were treated with MnTMPyP every day for 5 days prior to subjecting them to IH conditions. As shown in Fig. 5B, MnTMPyP treatment completely prevented IH-evoked facilitation of hypoxia-induced CA secretion in terms of both the number of secretory events and the amount of CA secreted per event. The remaining secretory response to hypoxia was comparable with that of control cells (Fig. 5C). Also, MnTMPyP prevented IH-induced facilitation of the [Ca\(^{2+}\)]i response to hypoxia as well as the elevated norepinephrine and epinephrine contents of adrenal medullae (Fig. 6).

Similar results were also obtained with NAC (800 mg·kg\(^{-1}\)·day\(^{-1}\), ip for 5 days), another structurally and functionally distinct antioxidant. Thus in cells derived from IH pups treated with NAC, the number of secretory events/min and average CA molecules secreted per event (×10\(^5\)) during the hypoxic challenge were 53 ± 4.2/min and 4.6 ± 0.3/event, respectively (n = 12), which were similar to hypoxia-evoked secretory response in control cells [the number of secretory events/min = 43 ± 8; CA molecules/event (×10\(^5\)) = 5 ± 0.8; n = 17; P > 0.05].

**Effects of neonatal IH on chromaffin cells persist into adult life**

The effects of IH on carotid body response to hypoxia in adult rats could be completely reversed following re-exposing IH-treated rats to normoxia (Peng et al. 2003). To determine whether the effects of neonatal IH could be reversed following re-exposure to normoxia, rat pups were exposed to IH from P0 to P5 and were then reared under normoxia for 30 days. Subsequently, chromaffin cell responses to hypoxia (PO\(_2\) 30 mmHg) were determined in P35 rats. Control experiments were performed on age-matched rats that were not exposed to neonatal IH. In control P35 rats, only 20% of cells (6 of 30) responded to hypoxia with increased CA secretion, whereas in rats exposed to neonatal IH, nearly 45% of cells (11 of 24) responded to hypoxia. The magnitude of hypoxia-evoked CA

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**FIG. 6.** Antioxidant treatment prevents IH-evoked changes in [Ca\(^{2+}\)]i and CA contents. A: traces of mean [Ca\(^{2+}\)]i responses to hypoxia (PO\(_2\) 30 mmHg) determined every 2 s in individual chromaffin cells from control and IH or IH + MnTMPyP (antioxidant) treated chromaffin cells. Horizontal black bars represent the duration of the hypoxic challenge. The data presented for Control and IH were the same as in Fig. 3. B: average data of basal and hypoxia-evoked [Ca\(^{2+}\)]i changes in chromaffin cells from IH and IH + MnTMPyP-treated rat pups presented as percentage of control cells (Control = 100%). The data presented are means ± SE. Control = 33, IH = 38, and IH + MnTMPyP = 34 cells from 3 different litters in each group. **P < 0.001. C: average results showing the effects of antioxidant (MnTMPyP) treatment on IH-evoked up-regulation of norepinephrine (NE) and epinephrine (E) contents of adrenal medullae. CA contents were presented as percentage of controls (normoxia; Control = 100%). Data presented are means ± SE from 6 rat pups from 3 different litters in each group. **P < 0.001.
secretion (the number of secretory events as well as the amount of CA secreted per event) and elevations in $[Ca^{2+}]_{i}$ were significantly greater in neonatal IH-treated chromaffin cells compared with corresponding age-matched controls ($P < 0.001$; Fig. 7, A–D). In addition, the levels of MDA as well as norepinephrine and epinephrine in adrenal medullae were also significantly higher in P35 rats that were exposed to neonatal IH compared with controls ($P < 0.001$; Fig. 7E).

**SH attenuates hypoxia-evoked CA secretion**

To assess the effects of SH, rat pups were exposed to hypobaric hypoxia (0.4 atm) for either 24 h (P0–P1) or 5 days (P0–P5), after which the effects of acute hypoxia on CA secretion from chromaffin cell were determined. Control experiments were performed on age-matched pups reared under normoxia. Exposing rat pups to 24 h of SH had no significant effect on hypoxia-evoked CA secretion compared with controls [Number of events/min: Control = 44 ± 6 vs. SH = 48 ± 9; average CA molecules/event ($10^{5}$); Control = 5 ± 0.9 vs. SH = 5.8 ± 1; SH vs. Control: $P > 0.05$; $n = 12$ cells each]. On the other hand, after 5 days of SH, hypoxia-evoked CA secretion was reduced, which primarily arose from a decrease in the amount of CA released per secretory event ($P < 0.01$; Fig. 8). Furthermore, the number of cells responding to hypoxia decreased in pups treated with 5 days of SH (Control = 17 of 23 cells = 74% vs. SH = 18 of 32 cells = 56%). Likewise, SH treatment attenuated $K^+$-evoked CA secretion, which also arose from a reduced amount of CA released per secretory event (SH = 5 ± 0.2 vs. Control = $7 ± 0.5 \times 10^5$ CA molecules per event; $P < 0.01$; $n = 20$ and 16 cells for SH and control, respectively). The levels of basal as well as the magnitude of hypoxia-induced elevation in $[Ca^{2+}]_{i}$ were comparable between SH and control cells ($P > 0.05$). Furthermore, SH had no significant effect on norepinephrine, epinephrine, and MDA levels of the adrenal medullae compared with controls ($P > 0.05$; control and SH, $n = 6$ adrenal medulla in each group).

**DISCUSSION**

Major findings of the present study were: 1) exposure of neonatal rat pups to IH for periods as short as 5 days led to pronounced facilitation of hypoxia-evoked CA secretion, elevation of $[Ca^{2+}]_{i}$ in chromaffin cells, and up-regulation of CA content in the adrenal medulla; 2) IH increased ROS levels in adrenal medullae and antioxidant treatment prevented IH-evoked facilitation of CA secretion, changes in $[Ca^{2+}]_{i}$, and up-regulation of CA content; 3) the effects of neonatal IH on chromaffin cells persisted into adult life; and 4) SH attenuated...
hypoxia-evoked CA secretion from chromaffin cells and this effect was associated with no significant changes in hypoxia-induced elevation in [Ca\(^{2+}\)]_i, CA content, and ROS levels in adrenal medulla.

Consistent with the previous studies on the carotid body (Pawar et al. 2008; Peng and Prabhakar 2004), we also found that IH enhances hypoxic sensing of chromaffin cells from neonatal rat pups. A previous study reported that prior exposure to IH appreciably facilitates hypoxia-evoked CA efflux from adult rat adrenal medulla (Kumar et al. 2006). However, it was not clear whether this IH-induced CA efflux was due to augmented CA secretion or/and inhibition of CA uptake. Using the amperometric approach, the current study demonstrates that antioxidant prevented this response suggests that ROS acts due not only to enhanced number of secretory events but also to the elevated neurotransmitter content of each event. The following observations demonstrate that ROS signaling mediates the facilitatory effects of IH on neonatal chromaffin cells. First, IH increased ROS in adrenal medulla as evidenced by elevated MDA levels. Second, antioxidants prevented IH-evoked facilitation of CA secretion by hypoxia. Previous studies suggest that depolarization (Garcia-Fernandez et al. 2007; Mojet et al. 1997; Thompson et al. 1997) and subsequent activation of voltage-gated Ca\(^{2+}\) channels and the ensuing elevations in [Ca\(^{2+}\)]_i near the site of secretory vesicles. The imaging technique used in the current study is due to the lack of an increase in [Ca\(^{2+}\)]_i, near the site of secretion.

Unlike adrenal medulla from adult rats (Hui et al. 2003), IH increased both norepinephrine and epinephrine contents in neonatal adrenal medulla. We believe that the elevated CA content represents increased synthesis because IH activates tyrosine hydroxylase, the rate-limiting enzyme in CA synthesis (Kumar et al. 2003). The observation that antioxidant treatment prevents IH-evoked up-regulation of CA suggests that in addition to Ca\(^{2+}\) signaling, ROS-mediated activation of CA synthesis also contributes to facilitation of CA secretion by hypoxia.

An intriguing finding of the present study was that although IH was given for only 5 days in neonatal life, its effects persisted even into adult life. We believe that the long-lasting effects of neonatal IH are due to elevations of ROS levels, which might be attributed to long-term changes in genes...
associated with the maintenance of cellular redox state. It is being increasingly appreciated that epigenetic regulation via DNA methylation and histone modifications, especially during development, leads to long-term changes in gene expression (Feinberg 2007). The sustained elevations in ROS levels could conceivably be due to epigenetic regulation of genes encoding either pro- and/or antioxidant enzyme(s) by IH, a possibility that requires further study.

Prolonged exposure to SH facilitates hypoxia-evoked CA secretion in rat PC12 cells (Taylor and Peers 1999) and elevates ROS levels in cell cultures (Bell et al. 2007; Guzy et al. 2005). Based on these studies, we anticipated that SH would also facilitate hypoxia-induced CA secretion from neonatal chromaffin cells via ROS signaling. Contrary to our expectation, SH attenuated hypoxia-evoked CA secretion due to a reduction in the amount of CA released per secretory event. The effects of SH on CA secretion were associated with un-altered ROS levels in adrenal medullae. The differences between our results and those reported previously on cell cultures (Bell et al. 2007; Guzy et al. 2005; Taylor and Peers 1999) could arise from differences in the experimental preparations such as cell culture versus intact animals as well as propagating phenotype of cells used in earlier studies versus the nonpropagating nature of native chromaffin cells used in this study. Hypobaric hypoxia can lower the metabolism and body temperature in rat pups (Baig and Joseph 2008). Alternatively, the reduced CA secretion might be secondary to the effects of SH on body metabolism, resulting in reduced tyrosine hydroxylase activity leading to decreased CA synthesis. The attenuated secretory response to hypoxia appears unlikely due to changes in CA synthesis because neither norepinephrine nor epinephrine levels were altered in SH-treated compared with control adrenal medullae. It is possible that reduced emptying of the secretory vesicles might account for the attenuated CA release. In chromaffin cells, weak stimulation leading to modest elevation in [Ca\(^{2+}\)]\(_i\) tends to promote only a partial release of the vesicular CA content via a kiss-and-run mechanism, whereas stronger stimuli resulting in robust elevation in [Ca\(^{2+}\)]\(_i\), either evokes a more complete emptying of the vesicle content or, alternatively, causes vesicles to undergo full fusion (Elhamdani et al. 2001, 2006). The absence of facilitation of hypoxia-evoked CA secretion might in part be due to the inability of SH to augment hypoxia-evoked [Ca\(^{2+}\)]\(_i\) response, which is in part attributed to the absence of enhanced ROS generation by SH.

In summary, the present study demonstrates that exposing neonatal rat pups to IH facilitates, whereas to SH attenuates, CA secretion by hypoxia. The effects of neonatal IH persisted even into adult life. What might be the significance of long-lasting up-regulation of the hypoxic sensitivity of chromaffin cells by neonatal IH? Between 70 and 90% of prematurely born infants experience IH because of recurrent apneas (Stokowski 2005), with each episode lasting two breaths or longer. It is likely that premature infants experiencing IH as a consequence of recurrent apneas are vulnerable to develop cardiovascular morbidities in adult life because of the augmented CA secretion by hypoxia, which is a common stress that is encountered under a variety of physiological and pathophysiological situations.

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