Chronic intermittent hypoxia induces hypoxia-evoked catecholamine efflux in adult rat adrenal medulla via oxidative stress

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Chronic intermittent hypoxia (CIH) augments physiological responses to low partial pressures of O_2 in the arterial blood. Adrenal medullae from adult rats, however, are insensitive to direct effects of acute hypoxia. In the present study, we examined whether CIH induces hypoxic sensitivity in the adult rat adrenal medulla and, if so, by what mechanism(s). Experiments were performed on adult male rats exposed to CIH (15 s of 5% O_2 followed by 5 min of 21% O_2; 9 episodes h^{-1}; 8 h d^{-1}; for 3 or 10 days) or to comparable, cumulative durations of continuous hypoxia (CH; 4 h of 7% O_2 followed by 20 h of 21% O_2 for 1 or 10 days). Noradrenaline (NA) and adrenaline (ADR) effluxes were monitored from *ex vivo* adrenal medullae. In adrenal medullae of rats exposed to CIH, acute hypoxia evoked robust NA and ADR effluxes, whereas these responses were absent in control rats or in those exposed to CH for 1 or 10 days. Hypercapnia (10% CO_2; either acidic, pH 6.8, or isohydric, pH 7.4) was ineffective in eliciting catecholamine (CA) efflux from control, CIH or CH rats. Nicotine (100 μM) evoked NA and ADR effluxes in control rats, and this response was abolished in CIH but not in CH rats. Systemic administration of 2-deoxyglucose depleted ADR content in control rats, and CIH attenuated this response, indicating downregulation of neurally regulated CA secretion. Cytosolic and mitochondrial aconitase enzyme activities decreased in CIH adrenal medullae, suggesting increased generation of superoxide anions. Systemic administration of antioxidants reversed the effect of CIH on the adrenal medulla. Rats exposed to CIH exhibited increased blood pressures and elevated plasma CA, and antioxidants abolished these responses. These observations demonstrate that CIH induces hypoxic sensing in the adult rat adrenal medulla via mechanisms involving increased generation of superoxide anions and suggest that hypoxia-evoked CA efflux from the adrenal medulla contributes, in part, to elevated blood pressure and plasma CA.

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Humans with sleep-disordered breathing (i.e. obstructive or central apnoeas) experience chronic intermittent hypoxia (CIH). Previous studies have reported elevated blood pressure and increased plasma as well as urinary catecholamines (CA) in sleep apnoea patients and in experimental animals exposed to CIH (Fletcher *et al.* 1987; Fletcher & Bao, 1996; Phillips & Somers, 2000). Adrenalectomy prevents elevation in blood pressure and increases in plasma CA in rats exposed to CIH, suggesting that CA secretion from the adrenal medulla plays a critical role in eliciting cardiovascular changes (Bao *et al.* 1997). In adult animals, hypoxia-evoked adrenal CA secretion is neurogenic and requires activation of the sympathetic nervous system (Seidler & Slotkin, 1986; Yokotani *et al.* 2002), whereas adult adrenal medullae are relatively insensitive to direct effects of acute hypoxia (Thompson *et al.* 1997; Keating *et al.* 2001). We previously reported that CIH selectively enhances hypoxic sensing by the carotid bodies (Peng & Prabhakar, 2004) and augments hypoxia-evoked transmitter release in cell cultures (Kim *et al.* 2004). Although these studies suggest that hypoxic sensing is facilitated by CIH, it is not known whether CIH also induces hypoxic sensing in the adult adrenal medulla. Therefore, in the present study, using an *ex vivo* preparation, we determined whether acute hypoxia evokes CA efflux from the adrenal medulla of adult rats exposed to CIH and, if so, by what mechanism(s). Our results demonstrate that CIH, but not a comparable,

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cumulative duration of continuous hypoxia (CH), induces CA efflux in response to acute hypoxia, and the effects of CIH are associated with increased oxidative stress.

Methods

Exposure to CIH

The animal handling procedures and the experimental protocols were approved by the Institutional Animal Care and Use Committee of the Case Western Reserve University. Adult, male Sprague–Dawley rats weighing 250–300 g were exposed to 3 and 10 days of CIH, i.e. alternating cycles of hypoxia (5% O_2 in N_2 for 15 s) and normoxia (21% O_2 in N_2 for 5 min), 9 episodes h^{-1} and 8 h day^{-1}, as previously reported (Peng *et al.* 2003). To determine the effect of a comparable, cumulative duration of continuous hypoxia (CH), rats were exposed to 4 h of 7% O_2 in N_2 followed by 20 h of room air for 1 or 10 days. The 4 h of hypoxia is equivalent to the hypoxic duration accumulated during 10 days of CIH. Rats exposed to 10 days of room air (normoxia) served as controls. In experiments wherein the effects of antioxidants were examined, rats were given either manganese (III) tetrakis(1-methyl-4-pyridyl) porphyrin pentachloride (MnTMPyP; ALEXIS Biochemicals, Carlsbad, CA, USA; 5 mg kg^{-1} day^{-1} I.P.), which is a superoxide dismutase (SOD) mimetic, or *N*-acetylcysteine (NAC; 800 mg kg^{-1} day^{-1} I.P.) or vehicle (500 μl saline, I.P.) every day for 10 days before exposing them to CIH. Acute experiments were performed on rats anaesthetized by an intraperitoneal injection of urethane (1.2 g kg^{-1}, Sigma). Supplemental doses of anaesthetics were given when corneal reflexes and responses to toe pinch persisted. Acute experiments were conducted ~12 h following either CIH or CH exposures. At the end of the experiment, rats were killed by intracardiac injection (0.1 ml) of euthanasia solution (Beuthanasia-D Special, (each ml contains 390 mg pentobarbital sodium and 50 mg phenytain sodium) Schering-Plough Animal Health, Kenilworth, NJ, USA).

Measurement of catecholamines

Experiments were performed on freshly harvested adrenal medullae from anaesthetized rats. Tissues were homogenized and CA were extracted with 0.1 N $HClO_4$ containing 10 mM EDTA-Na_2 and assayed by high performance liquid chromatography coupled with electrochemical detection (HPLC-ECD) method as previously described (Kumar *et al.* 1998). Noradrenaline (NA) and adrenaline (ADR) were eluted at 2.8 and 4.2 min, respectively.

In the first series of experiments ($n = 8$ rats in each group), the effect of acute hypoxia and nicotine on CA efflux were assessed. To stabilize the basal efflux, adrenal medullae were incubated sequentially three times in Krebs Ringer bicarbonate medium equilibrated at normoxia (21% O_2 + 5% CO_2 + N_2; partial pressure of O_2, $P_{O_2} = 146 \pm 6$ mmHg) and then challenged with either acute hypoxia (1% O_2 + 5% CO_2 + N_2; $P_{O_2} = 35 \pm 4$ mmHg) or nicotine (100 μM) for 5 min each. The P_{O_2} of the medium was measured with a blood gas analyser (Radiometer ABL5, Copenhagen, The Netherlands). Preliminary experiments with varying concentrations of nicotine (10, 30, 100 and 300 μM) showed that maximal CA effluxes could be elicited from *ex vivo* adrenal medulla with 100 μM of nicotine during 5 min of incubation. Prolonged incubations, however, resulted in progressive decreases in NA and ADR effluxes. Therefore, all subsequent studies were performed with 5 min incubation of tissues with 100 μM nicotine.

In the second series of experiments, the effects of either acidic hypercapnia (21% O_2 + 10% CO_2 + N_2, pH 6.8) or isohydric hypercapnia (21% O_2 + 10% CO_2 + N_2, pH 7.4) on CA efflux were determined ($n = 8$ rats in each group). For isohydric hypercapnia, the extracellular pH was maintained at 7.4 by the addition of 44 mM of HCO_3^-. Noradrenaline and ADR effluxes were expressed as picomoles per minute per adrenal medulla.

Measurement of plasma catecholamines

Arterial blood samples were collected in heparinized vials (heparin, 30 i.u. ml^{-1}) from anaesthetized rats. Plasma was separated and CA extracted with acid-activated alumina. Noradrenaline and ADR were determined by HPLC-ECD as described above. 3,4-Dihydroxybenzylamine was used as an internal standard, and the recoveries for NA and ADR were ~78 and 80%, respectively. Catecholamine values were expressed as nanograms of NA or ADR per 100 ml of plasma. Two series of experiments were performed. In one series, the effect of acute hypoxia (12% O_2 for 2 min) on plasma ADR and NA was examined, whereas in another series, the effect of antioxidants on plasma CA was determined in control and CIH rats ($n = 8$ rats in each group).

Effect of 2-deoxyglucose (2-DG) on adrenal medullary catecholamine content

Control and CIH-treated rats were given either 2-deoxyglucose (1.5 g (kg body weight)$^{-1}$ I.P.; Lau *et al.* 1987) or saline ($n = 6$ rats in each group). After 3 h, adrenal medullae were removed and ADR and NA contents determined by HPLC-ECD as described above.

Measurement of blood pressure

Arterial blood pressure was determined via a femoral artery catheter connected to a blood pressure

transducer (Grass Model PT300; West Warwick, RI, USA) in anaesthetized, spontaneously breathing rats exposed either to normoxia or to 10 days of CIH ($n = 12$ rats in each group).

Measurement of aconitase activity

Aconitase enzyme activity was determined in cytosolic and mitochondrial fractions of adrenal medulla as previously described (Gardner *et al.* 1995) and expressed as micromoles of isocitrate per minute per milligram of protein.

Data analysis

All data are expressed as means ± S.E.M. Statistical significance was evaluated by Student's unpaired *t* test or one-way ANOVA for repeated measures. *P* values less than 0.05 were considered significant.

Results

Effect of CIH on hypoxia-evoked CA efflux

Exposure to CIH for either 3 or 10 days resulted in a significant increase in basal CA efflux from adrenal medullae compared to normoxic control rats. Chronic intermittent hypoxia increased NA efflux by 94% (CIH, 123 ± 13 *versus* control, 63 ± 4 pmol min^{-1} tissue^{-1}) and ADR efflux by 42% (CIH, 537 ± 56 *versus* control, 385 ± 24 pmol min^{-1} tissue^{-1}; $P < 0.01$, $n = 8$ rats in each group). Tissue contents of ADR and NA were determined to assess whether the CIH-induced increase in basal CA efflux resulted from increased tissue content. Following CIH, NA content increased by ~68% (CIH, 25.2 ± 4 *versus* control, 15 ± 3 nmol tissue^{-1}; $P < 0.01$, $n = 8$ in each group), whereas ADR levels were unaltered (CIH, 43 ± 2 *versus* control, 44 ± 2 nmol tissue^{-1}; n.s., $n = 8$ in each group).

Acute hypoxia ($P_{O_2} = 35 \pm 4$ mmHg) had no significant effect on NA and ADR effluxes in control adrenal medullae (n.s., $n = 8$; Fig. 1). In contrast, prior exposure to CIH significantly facilitated CA efflux in response to acute hypoxia. The effect of CIH on the acute hypoxic response was, however, time dependent. Exposure to 3 days of CIH had no effect, whereas 10 days of CIH resulted in ~2.8- and 2.6-fold increases in NA and ADR effluxes, respectively, in response to acute hypoxia (control *versus* CIH, $P < 0.01$, $n = 8$ in each group; Fig. 1).

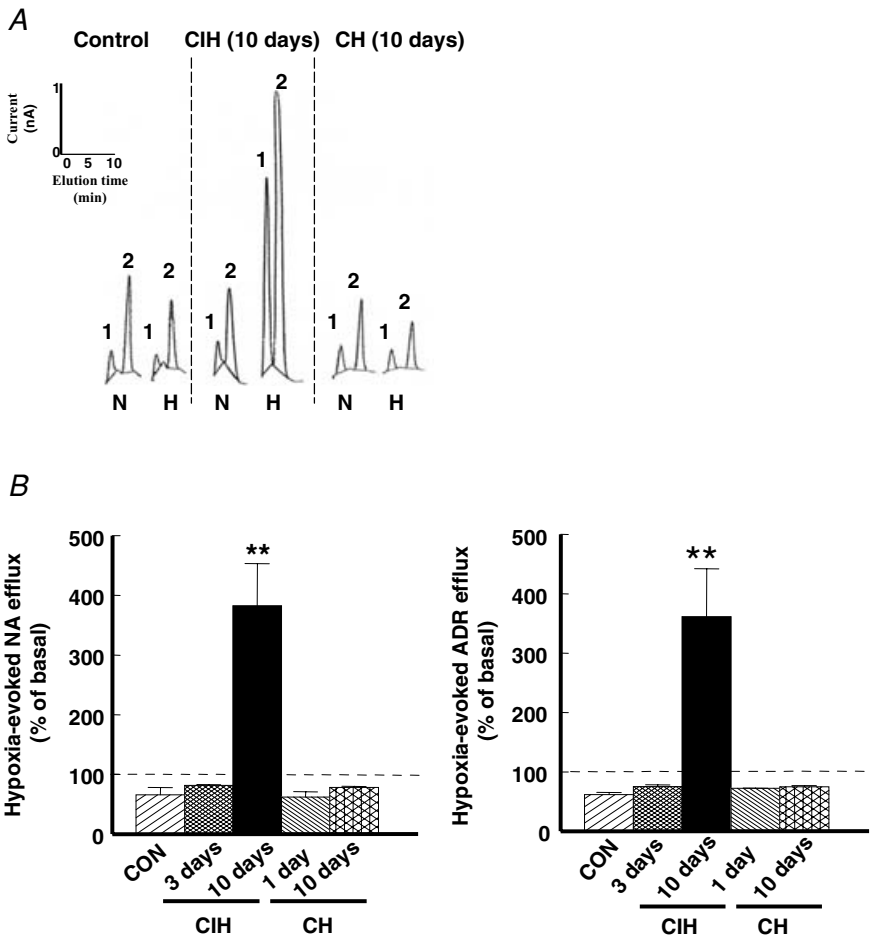

Figure 1. Effect of acute hypoxia on NA and ADR effluxes in adrenal medullae of rats exposed to normoxia, CIH and CH
Adrenal medulla of adult rats exposed to 10 days of normoxia (CON), chronic intermittent hypoxia (CIH) and a comparable, cumulative duration of continuous hypoxia (CH) were incubated with either normoxic (medium P_{O_2}, 146 ± 6 mmHg) or hypoxic medium (medium P_{O_2}, 35 ± 4 mmHg) for 5 min, as described in the Methods. Representative HPLC elution profiles of NA and ADR effluxes in response to normoxia (N) and hypoxia (H) in the control, CIH (10 days) and CH (10 days) rat adrenal medulla are shown in *A*. Peaks 1 and 2 represent the elution of NA and ADR, respectively. Average data on the effect of acute hypoxia on NA and ADR effluxes are shown in *B*, left and right panels, respectively. The data in *B* are expressed as a percentage of basal normoxic efflux (= 100%). The basal effluxes of NA and ADR, in pmol min^{-1} tissue^{-1}, were as follows: for CON, NA = 63 ± 4 and ADR = 385 ± 24; for CIH, NA = 123 ± 13 and ADR = 537 ± 56; and for CH, NA = 45 ± 5 and ADR = 239 ± 29. Results represent means ± S.E.M. from 8 rats in each group; $^{**}P < 0.01$ compared to CON.

Effect of CH on hypoxia-evoked CA efflux

To determine whether a comparable, cumulative duration of CH also facilitates CA efflux in response to acute hypoxia, rats were exposed to 4 h of CH, a duration of hypoxia which is equivalent to that accumulated during 10 days of CIH. Unlike CIH, CH resulted in a significant decrease of basal CA efflux ($P < 0.01$, $n = 8$). Noradrenaline efflux decreased by 31% (CH, 45 ± 5 *versus* control, 63 ± 4 pmol min^{-1} tissue^{-1}) and ADR by 40% (CH, 239 ± 29 *versus* control, 385 ± 24 pmol min^{-1} tissue^{-1}). Continuous hypoxia, however, had no facilitatory influence on NA and ADR effluxes in response to acute hypoxia (n.s., $n = 8$; Fig. 1*B*). It is possible that a single exposure to CH may not be adequate in facilitating the hypoxic response. Therefore, to test whether multiple exposures to CH can induce hypoxic sensitivity, another group of rats were exposed to 4 h of hypoxia per day for 10 days. As with single exposure, multiple exposures to CH also decreased basal CA efflux and had no facilitatory effect on CA efflux in response to acute hypoxia (n.s., $n = 8$; Fig. 1*B*).

The following experiments were performed on rats exposed to 10 days CIH, since they exhibited significant facilitation of the hypoxic response of the adrenals.

Effect of CIH and CH on CA efflux induced by hypercapnia

Hypercapnia (acidic and isohydric) evokes CA efflux from neonatal but not from adult rat adrenal medulla (Munoz-Cabello *et al.* 2005; Rico *et al.* 2005). To determine whether the effects of CIH are confined to hypoxia or extend to other stimuli, the effects of acidic hypercapnia (pH 6.8, 10% CO_2) on CA efflux were determined. In the control adrenal medullae, acidic hypercapnia had no significant effect on either NA (basal, 63 ± 4 *versus* acidic hypercapnia, 51 ± 11 pmol min^{-1} tissue^{-1}; n.s., $n = 8$) or ADR efflux (basal, 385 ± 24 *versus* acidic hypercapnia, 308.5 ± 48 pmol min^{-1} tissue^{-1}; n.s., $n = 8$). Unlike hypoxia, CIH did not facilitate CA efflux by acidic hypercapnia (NA: basal, 123 ± 13 *versus* acidic hypercapnia, 98.6 ± 21 pmol min^{-1} tissue^{-1}; ADR: basal, 537 ± 56 *versus* acidic hypercapnia, 445 ± 76 pmol min^{-1} tissue^{-1}; n.s., $n = 8$). Likewise, adrenal medullae from rats exposed to 10 days of CH also did not respond to acidic hypercapnia with facilitated CA efflux (NA: basal, 45 ± 5 *versus* acidic hypercapnia, 38 ± 10 pmol min^{-1} tissue^{-1}; ADR: basal, 239 ± 29 *versus* acidic hypercapnia, 217 ± 32 pmol min^{-1} tissue^{-1}; n.s., $n = 8$).

In another series of experiments, the effect of isohydric hypercapnia (pH 7.4, 10% CO_2) on CA efflux was determined. In the control adrenal medullae, isohydric hypercapnia had no facilitatory effect on CA efflux (NA: basal, 63 ± 4 *versus* isohydric hypercapnia, 53 ± 11 pmol min^{-1} tissue^{-1}; ADR: basal, 385 ± 24 *versus* isohydric hypercapnia, 329 ± 39 pmol min^{-1} tissue^{-1}; n.s., $n = 8$). The lack of isohydric hypercapnia-evoked CA efflux persisted after 10 days of CIH (NA: basal, 123 ± 13 *versus* isohydric hypercapnia, 106 ± 17 pmol min^{-1} tissue^{-1}; ADR: basal, 537 ± 56 *versus* isohydric hypercapnia, 512 ± 36 pmol min^{-1} tissue^{-1}; n.s., $n = 8$ in each group). Similarly, exposure to 10 days of CH also had no facilitatory effect on CA effluxes in response to isohydric hypercapnia (NA: basal, 45 ± 5 *versus* isohydric hypercapnia, 39 ± 9 pmol min^{-1} tissue^{-1}; ADR: basal, 239 ± 29 *versus* isohydric hypercapnia 210 ± 28 pmol min^{-1} tissue^{-1}; n.s., $n = 8$ in each group).

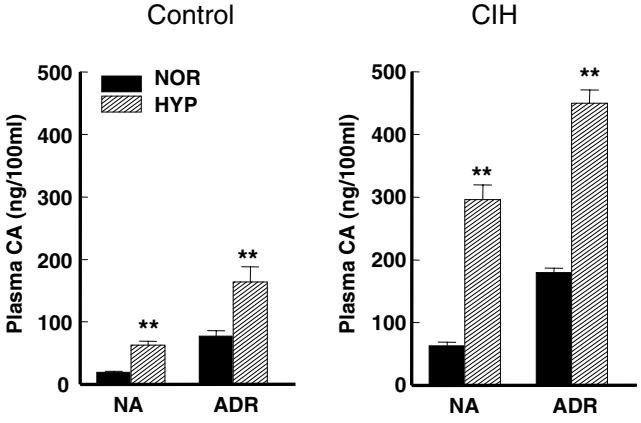

Figure 2. Chronic intermittent hypoxia facilitates acute hypoxia-evoked increase in plasma CA
Plasma catecholamine (CA) levels in the control (left panel) and CIH-exposed rats (right panel) were measured by HPLC-ECD as described in the Methods. Results represent means ± S.E.M. from 8 rats in each group; $^{**}P < 0.01$ compared to normoxia (NOR). HYP, hypoxia.

Effect of acute hypoxia on plasma CA

The experiment described above using *ex vivo* adrenal medullae suggests that CIH facilitates CA efflux in response to acute hypoxia. To further establish the functional relevance of the above findings, we examined the effect of CIH on plasma NA and ADR effluxes in response to acute hypoxia (12% O_2 for 2 min) in anaesthetized, spontaneously breathing rats. The results are summarized in Fig. 2. In control rats, acute hypoxia increased plasma NA from 19 ± 2 (basal) to 63 ± 6 ng (100 ml)$^{-1}$ and ADR from 77 ± 9 (basal) to 164 ± 24 ng (100 ml)$^{-1}$ ($P < 0.01$; $n = 8$; Fig. 2, left panel). In CIH conditioned animals, basal plasma NA and ADR were 63 ± 6 and 180 ± 7 ng (100 ml)$^{-1}$, respectively, which were significantly higher than the equivalent values in control rats ($P < 0.01$; $n = 8$). Acute hypoxia further elevated plasma NA and ADR to 296 ± 24 and

450 ± 21 ng $(100\ \text{ml})^{-1}$, respectively (Fig. 2, right panel). Thus, in CIH rats, acute hypoxia evoked a greater increase in plasma NA ($+148 \pm 7\%$) and ADR ($+38 \pm 5\%$) than in the control rats (CIH *versus* control; $P < 0.01$, $n = 8$ in each group; Fig. 2).

Chronic intermittent hypoxia attenuates nicotine-evoked CA efflux

Nicotine (100 μM) evoked five- and threefold increases in NA and ADR effluxes, respectively, from the control adrenal medullae ($P < 0.01$, $n = 8$; Fig. 3*A* and *B*). Chronic intermittent hypoxia abolished or attenuated nicotine-evoked CA efflux in a time-dependent manner. Thus, 3 days of CIH reduced nicotine-evoked NA efflux by 80% ($P < 0.01$, $n = 8$; Fig. 3*B*, left panel) whereas ADR efflux was completely abolished (Fig. 3*B*, right panel). In contrast, 10 days of CIH abolished nicotine-evoked efflux of both NA and ADR ($n = 8$, Fig. 3*A* and *B*). Unlike CIH, either single (data not shown) or multiple exposures to CH did not attenuate nicotine-evoked CA efflux (n.s., $n = 8$; Fig. 3).

The results described in the preceding paragraph demonstrate that nicotine-evoked CA efflux from adrenal medulla is abolished by CIH. Since activation of nicotinic cholinergic receptors is critical for evoking neurally mediated CA secretion from the adrenal medulla, we hypothesized that CIH downregulates neurogenic mediation of CA secretion. To test this possibility, we assessed the effect of systemic administration of 2-deoxyglucose (2-DG), which selectively depletes ADR from the adrenal medulla by activating 2-DG-sensitive sympathetic fibres (Kuzmin *et al.* 1995; Vollmer *et al.* 1997; Morrison & Cao, 2000). In control rats, 2-DG reduced adrenal ADR from 42 ± 0.5 to 9 ± 1.2 nmol per adrenal medulla (78% depletion; Fig. 4). In CIH rats, 2-DG caused a reduction in ADR content from 41 ± 2.3 to 20 ± 0.3 nmol per adrenal medulla (51% depletion). Thus, the magnitude of the 2-DG-induced depletion of ADR was significantly less in CIH than in control adrenal medulla ($P < 0.01$; $n = 6$ rats in each

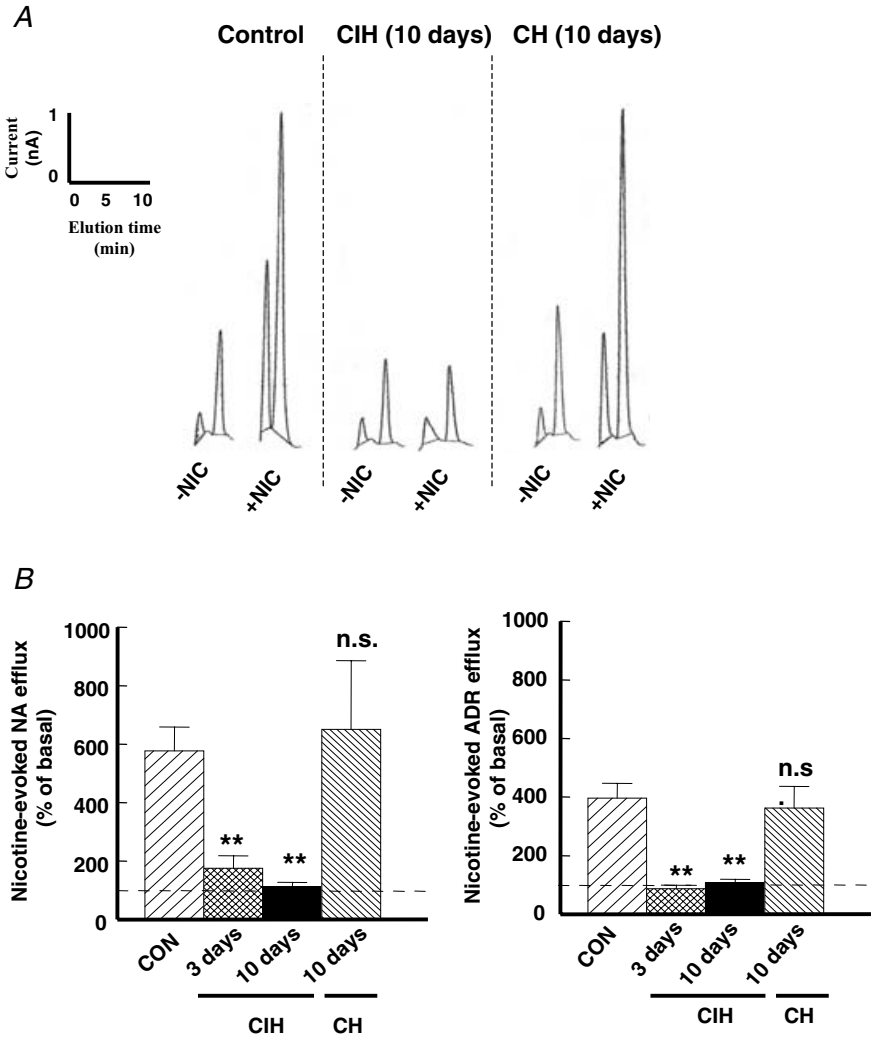

Figure 3. Comparison of the effect of nicotine on NA and ADR effluxes in the adrenal medulla of rats exposed to normoxia, CIH and CH
Adrenal medullae of rats exposed to 10 days of normoxia (CON), CIH and CH were incubated in normoxic medium (P_{O_2}, 146 ± 6 mmHg) with and without nicotine (100 μM) for 5 min as described in the Methods. Representative HPLC elution profiles of NA and ADR effluxes in response to nicotine in the control, CIH- (10 days) and CH-exposed rat (10 days) adrenal medulla are shown in *A*. The first and the second peak represent the elution of NA and ADR, respectively. −NIC, in the absence of nicotine; +NIC, in the presence of nicotine. Average data on the effects of nicotine on NA and ADR effluxes are shown in *B*, left and right panels, respectively. The data are expressed as a percentage of basal efflux (= 100%). The basal effluxes of NA and ADR, in pmol min^{-1} tissue^{-1}, are given in the legend of Fig. 1. Results represent means ± S.E.M. from 8 rats in each group. n.s., not significant.

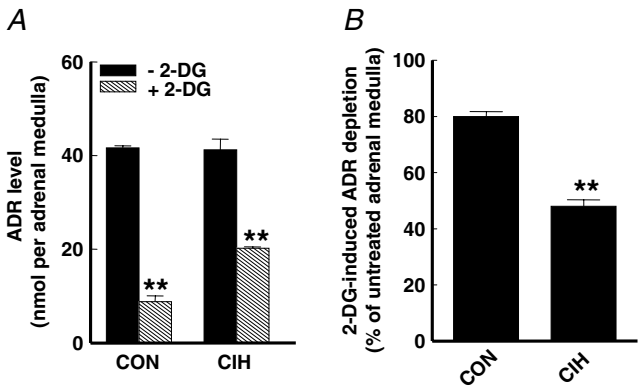

Figure 4. Chronic intermittent hypoxia attenuates 2-deoxyglucose-induced depletion of ADR in rat adrenal medulla
The effect of 2-DG (1.5 g (kg body weight)$^{-1}$) on ADR levels in the adrenal medulla of normoxic rats (CON) and rats exposed to CIH for 10 days are shown in *A*. The effect of CIH on 2-DG-induced ADR depletion is shown in *B*. The experimental details are as outlined in the Methods. Results represent means ± S.E.M. from 6 rats in each group. $^{**}P < 0.01$ compared to untreated control animals. Note that CIH significantly reduced 2-DG-induced ADR depletion from the adrenal medulla.

group; Fig. 4*B*). As reported by others (Kuzmin *et al.* 1995; Vollmer *et al.* 1997; Morrison & Cao, 2000), we also found that 2-DG had no significant effect on the NA content of normoxic and CIH adrenal medullae.

CIH increases $O_2^{\cdot-}$ in the adrenal medulla

In order to begin to examine the mechanisms associated with CIH-induced hypoxic sensitivity, we first examined whether CIH increases $O_2^{\cdot-}$ in the adrenal medulla, because $O_2^{\cdot-}$ anions have been implicated in CIH-induced functional changes in intact animals and in cell cultures (Prabhakar & Kumar, 2004). Aconitase enzyme activity was monitored in the cytosolic and mitochondrial fractions of the adrenal medulla as an index of $O_2^{\cdot-}$ generation (Gardner *et al.* 1995). Chronic intermittent hypoxia significantly decreased aconitase activity in cytosolic (52%) and mitochondrial fractions (79%; CON *versus* CIH, $P < 0.01$, $n = 6$ rats in each group; Fig. 5). MnTMPyP, a membrane-permeable superoxide dismutase (SOD) mimetic and a potent scavenger of $O_2^{\cdot-}$ anions, prevented CIH-induced inhibition of aconitase activity (CIH *versus* CIH + MnTMPyP, $P < 0.01$, $n = 6$ rats in each group; Fig. 5). In the control rats, however, MnTMPyP had no significant effect on aconitase enzyme activity either in the mitochondrial or the cytosolic fractions (n.s., $n = 6$).

Superoxide anions ($O_2^{\cdot-}$) are critical for induction of hypoxic sensitivity by CIH

If $O_2^{\cdot-}$ anions contribute to CIH-induced hypoxic sensitivity, then antioxidants should prevent the effects of CIH. To test this possibility, rats were treated with either MnTMPyP or *N*-acetylcysteine (NAC, a precursor for glutathione and a potent scavenger of reactive oxygen species), and then were exposed either to CIH or to normoxia. Neither MnTMPyP nor NAC had any effect on the basal NA and ADR effluxes from adrenal medulla of both control and CIH rats (n.s., $n = 8$). However, both antioxidants abolished hypoxia-evoked NA and ADR effluxes from CIH adrenal medullae (CIH *versus* CIH + antioxidants, $P < 0.01$, $n = 8$ rats in each group; Fig. 6*A*). Furthermore, antioxidants reversed CIH-induced down-regulation of nicotine-evoked NA and ADR effluxes (CIH *versus* CIH + antioxidants, $P < 0.01$, $n = 8$ rats in each group; Fig. 6*B*).

Effect of antioxidants on CIH-induced elevations in blood pressure and plasma CA

We examined the effects of CIH on blood pressure and plasma CA and further determined whether antioxidants

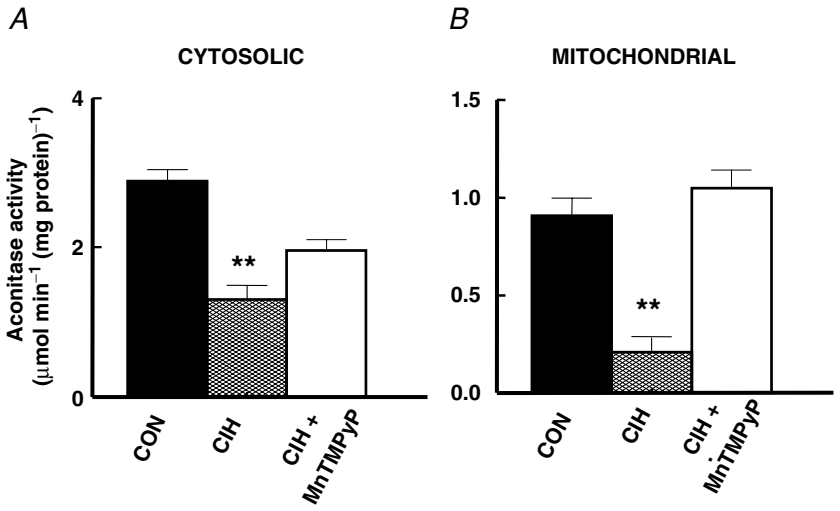

Figure 5. Chronic intermittent hypoxia inhibits aconitase activity in the cytosolic and mitochondrial fractions of the rat adrenal medulla
Cytosolic and mitochondrial fractions were prepared from adrenal medulla of control rats (CON) and rats exposed to CIH, with (CIH + MnTMPyP) and without (CIH) the SOD mimetic, MnTMPyP (5 mg kg^{-1} day^{-1} I.P., for 10 days) as described in the Methods. Aconitase activity was expressed as micromoles of isocitrate formed min^{-1} (mg protein)$^{-1}$. Data are means ± S.E.M. from 6 rats in each group. $^{**}P < 0.01$ compared to control values. Note the significant decrease in cytosolic and mitochondrial aconitase activity in CIH-exposed adrenal medulla, and the partial recovery of aconitase activity induced by MnTMPyP in the cytosolic fraction, with complete recovery of aconitase activity in the mitochondrial fraction.

effected CIH-induced changes in the cardiovascular responses. In CIH rats, mean arterial blood pressure was significantly elevated compared to control rats (control, 113 ± 3 *versus* CIH, 124 ± 4 mmHg; $P < 0.05$, $n = 12$ rats in each group; Fig. 7*A*). This increase in blood pressure was associated with significant elevations in plasma NA and ADR ($P < 0.01$, $n = 8$; Fig. 7*B*-*C*). Both the increase in blood pressure and the elevation in plasma CA were significantly attenuated in CIH rats treated with MnTMPyP (Fig. 7). Similar attenuation of blood pressures and plasma NA and ADR were also seen in CIH rats treated with NAC ($P < 0.01$; $n = 6$).

Discussion

Major findings of the present study are: (1) CIH but not CH facilitates acute hypoxia-evoked CA efflux, and this effect was associated with downregulation of nicotine-evoked CA efflux; (2) CIH potentiates acute hypoxia-evoked increases in plasma CA; (3) CIH increases oxidative stress in the adrenal medulla; and (4) antioxidants prevent CIH-induced functional changes in the adrenal medulla as well as elevation in arterial blood pressure and increase in plasma CA levels. To our knowledge, these observations demonstrate for the first time that CIH induces hypoxic sensitivity in adult rat adrenal medulla, which has potential functional consequences as evidenced by significant alterations in blood pressure and plasma CA.

In the present study, we employed an *ex vivo* adrenal medullary preparation for examining the stimulus-evoked CA responses. We refer to CA responses as 'effluxes' rather than 'secretion' because they were measured in the incubation medium, which represents secretion as well as reuptake of CA, if any, by the tissue. Previous studies, in contrast, employed vascularly perfused *ex vivo* adrenal medulla to examine stimulus-evoked CA secretion (Malhotra *et al.* 1988; Lim *et al.* 2004). In our preparation, even after 90 min stabilization, basal CA efflux was higher than that reported in vascularly perfused preparations. As a consequence, the overall magnitude of

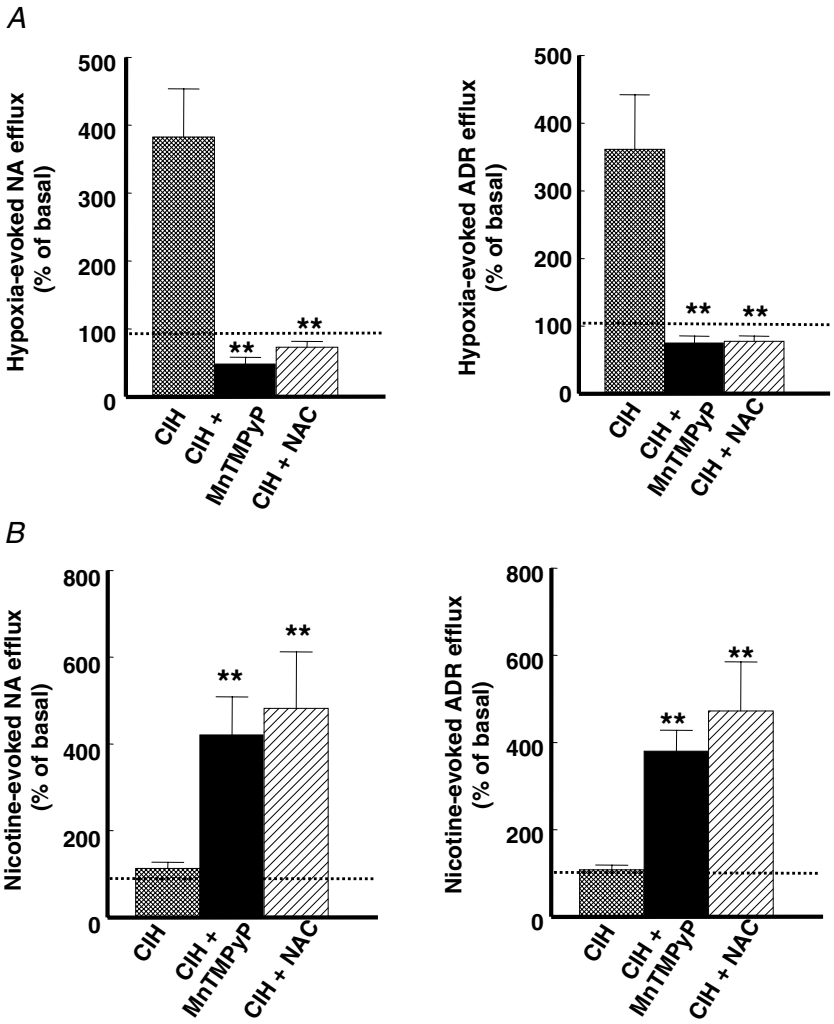

Figure 6. Effects of SOD mimetic and *N*-acetylcysteine (NAC) on CIH-induced alterations in stimulus-evoked NA and ADR effluxes in adrenal medulla
Effects of SOD mimetic (MnTMPyP) and NAC (800 mg kg^{-1} day^{-1} I.P.) on hypoxia-evoked (*A*) and 100 μM nicotine-evoked (*B*) NA (left panels) and ADR effluxes (right panels) from the adrenal medulla of rats exposed to CIH for 10 days are shown. The data are expressed as a percentage of basal efflux (= 100%). The basal effluxes of NA and ADR in pmol min^{-1} tissue^{-1} are given in the legend of Fig. 1. Superoxide dismutase mimetic treatment did not alter the basal effluxes of NA and ADR both in the control and CIH-exposed adrenal medullae. Note that MnTMPyP and NAC both prevented hypoxia-evoked CA efflux and restored nicotine responses in adrenal medulla of rats exposed to 10 days of CIH.

stimulus-evoked CA responses appears to be less than that previously reported (Malhotra *et al.* 1988; Lim *et al.* 2004). Nonetheless, consistent with previous studies (Seidler & Slotkin, 1986; Thompson *et al.* 1997), we also found that acute hypoxia had virtually no stimulatory effect on CA efflux in the control adrenal medulla from adult rats. Lack of the hypoxic response did not result from deterioration of the preparation because CA efflux could still be elicited by nicotine. In contrast to control rats, acute hypoxia consistently evoked both NA and ADR effluxes in CIH rats. These observations suggest that CIH induces hypoxic sensitivity in the adult rat adrenal medulla. However, it should be noted that the effect of CIH developed over time, in that hypoxic sensitivity was not apparent after 3 days but developed over a period of 10 days of CIH. The induction of hypoxic sensitivity is selective to CIH because either single or multiple exposures to comparable, cumulative duration of CH were ineffective in eliciting CA efflux in response to low P_{O_2}. It has been reported that hypoxic sensitivity of the carotid body can be selectively augmented by CIH but not by a comparable, cumulative duration of CH (Peng *et al.* 2003). The present results with adrenal medulla are consistent with these observations.

In intact animals exposed to CIH, acute hypoxia consistently evoked greater elevations in plasma ADR and NA compared to control animals. The enhanced plasma ADR levels in response to low P_{O_2} can be attributed to CIH-induced hypoxic sensitivity of the adrenal medulla because plasma ADR is primarily derived from the adrenal glands. Unlike ADR, plasma NA is derived not only from the adrenal medulla but also from sympathetic nerve terminals. Although CIH is known to augment sympathetic nerve response to acute hypoxia in rats (Fletcher *et al.* 1995) and in humans (Hardy *et al.* 1994; Smith *et al.* 1996; Cutler *et al.* 2004), we found that CIH attenuates neurally mediated CA efflux from adrenal medulla (see below). Therefore, we believe that the augmented plasma NA in response to acute hypoxia seen in rats exposed to CIH results from a direct effect of low P_{O_2} on adrenal NA efflux. These observations demonstrate that in rats exposed to CIH, the induction of hypoxic sensitivity of the adrenal medulla contributes to greater elevations in plasma CA in response to low P_{O_2}.

Are the effects of CIH selective to hypoxia? Recent studies reported that acidic and isohydric hypercapnia evoke CA efflux from neonatal but not from adult rat adrenal medullae (Munoz-Cabello *et al.* 2005; Rico *et al.* 2005). Hypercapnia being another physiological stimulus, we thought that CIH might also induce CO_2 sensitivity in adult adrenal medulla. Contrary to our expectation, hypercapnia (either acidic or isohydric) had virtually no effect on CA efflux in CIH adrenal medullae. These observations suggest that distinct mechanisms underlie CA efflux in response to hypoxia and hypercapnia, and CIH seems selectively to activate the former but not the latter mechanism.

What mechanism(s) underlie CIH-induced hypoxic sensitivity? Hypoxia exerts a direct effect on neonatal adrenal medulla, wherein sympathetic innervation is nearly absent. In contrast, in adult animals with intact sympathetic innervation, hypoxia no longer exerts a direct

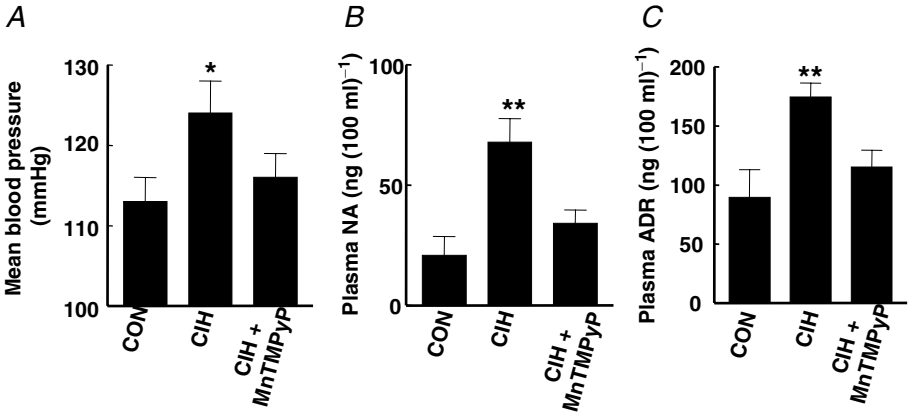

Figure 7. Superoxide dismutase mimetic reverses CIH-induced changes in mean blood pressure and plasma CA

Blood pressure was monitored in anaesthetized and spontaneously breathing rats, and plasma CA was measured by HPLC-ECD as described in the Methods. Average data for blood pressure and plasma NA and ADR are shown in *A*, *B* and *C*, respectively. CON, rats exposed to normoxia; CIH, rats exposed to 10 days of CIH; CIH + MnTMPyP, CIH-exposed rats treated with SOD mimetic (MnTMPyP) for 10 days (5 mg kg^{-1} day^{-1} I.P.). Data represent means ± S.E.M. from 12 rats in each group for blood pressure and for 8 rats in each group for plasma CA analysis. *$P < 0.05$, **$P < 0.01$ compared to controls. Note that CIH resulted in significant elevation in mean blood pressure and in plasma NA and ADR levels, and these changes were significantly attenuated or abolished in CIH-exposed rats treated with SOD mimetic.

stimulatory effect on the adrenal medulla (Thompson *et al.* 1997; Keating *et al.* 2001). Thus, there seems to be an inverse relation between hypoxic sensitivity of the adrenal medulla and sympathetic innervation. Supporting such a possibility are the observations that acute hypoxia evokes CA secretion from denervated adrenal medullae of adult animals (Keating *et al.* 2001) and from isolated chromaffin cells, which lack sympathetic innervation (Mochizuki-Oda *et al.* 1997; Thompson *et al.* 1997). The following observations from the present study demonstrate that CIH-induced hypoxic sensitivity is associated with functional denervation of the adrenal medulla. First, CIH induced a time-dependent downregulation of nicotinic responses, which are known to be important for neurogenic CA secretion from adrenal medulla (Yokotani *et al.* 2002) and to be correlated with induction of hypoxic sensitivity. Second, depletion of adrenal ADR by activation of 2-DG-sensitive sympathetic pathway(s) was attenuated in rats exposed to CIH. Third, in animals exposed to CIH, antioxidants restored the nicotinic response with a concomitant loss of hypoxic sensitivity (see below). Fourth, a comparable, cumulative duration of CH (either single or multiple exposures) neither downregulated nicotinic responses nor induced hypoxic sensitivity. It has been reported that CH reduces nicotinic responses in the carotid body (Jackson & Nurse, 1998). The fact that CH does not elicit similar effects in the adrenal medulla suggests that the effect of CH on the nicotinic response is tissue selective. The above findings, taken together, suggest that CIH-induced hypoxic sensitivity is coupled to functional denervation of the adrenal medulla as evidenced by downregulation of the nicotinic response. Whether CIH alters the expression, affinity and subunit composition of the nicotinic cholinergic receptor or downstream signalling mechanism(s) is beyond the scope of the present study and requires further investigation.

What are the upstream signalling events that trigger CIH-induced functional alterations in the adrenal medulla? Our data suggest that reactive oxygen species (ROS), especially the $O_2^{\cdot -}$ anion, are critical for inducing hypoxic sensitivity as well as abolishing nicotinic sensitivity brought about by CIH. First, aconitase activity, an established marker of $O_2^{\cdot -}$ (Gardner *et al.* 1994), is downregulated both in the cytosol and in the mitochondria of adrenal medulla of rats exposed to CIH. Second, MnTMPyP, a SOD mimetic, prevented CIH-induced inhibition of aconitase activity, further confirming that the decreased enzyme activity indeed resulted from increased generation of $O_2^{\cdot -}$. Third, and most important, is the evidence that antioxidants (MnTMPyP as well as NAC) not only prevented CIH-induced hypoxic sensitivity but also restored the sensitivity to nicotine in rats exposed to CIH. These observations suggest that ROS, particularly $O_2^{\cdot -}$, are upstream signalling molecules and are responsible for the CIH-induced hypoxic sensitivity and downregulation of nicotine sensitivity in the adult adrenal medulla.

What might be the source(s) of $O_2^{\cdot -}$ generation during CIH? Our previous studies on intact rats (Peng *et al.* 2003) and on cell cultures (Yuan *et al.* 2004) identified mitochondrial complex I as one of the sources of ROS generation by CIH. The fact that MnTMPyP treatment resulted in near complete restoration of mitochondrial aconitase activity in adrenal medullae from CIH-exposed rats suggests that mitochondria could be one of the major sources of ROS generation in response to CIH. Our results, however, do not exclude the potential contribution of cytosolic oxidases to the increased generation of ROS in response to CIH. In addition, further detailed investigation is required into whether CIH-induced effects result from $O_2^{\cdot -}$ and/or H_2O_2, a stable dismutated product of $O_2^{\cdot -}$, and into the mechanisms by which ROS lead to CIH-induced hypoxic sensitivity.

Antioxidants not only reversed the effects of CIH on adrenal medulla but, more importantly, they also attenuated or abolished CIH-induced increases in blood pressure and plasma CA. These findings suggest that increased CA efflux from adrenal medulla is of functional significance in that it contributes, at least in part, to elevated circulating CA, which in turn lead to increased blood pressure, either directly or via promoting the release of other vasoactive hormones. These observations suggest that oxidative stress resulting from increased ROS generation is an important mechanism that triggers CIH-induced cardiovascular changes, such as increased blood pressure.

In summary, the present study demonstrates that CIH induces hypoxic sensitivity in the adult rat adrenal medulla with a concomitant decrease in neurally evoked CA efflux. What might be the significance of such functional alterations? It is established that CIH increases sympathetic nerve activity (Greenberg *et al.* 1999; Fletcher, 2003; Prabhakar *et al.* 2005). Such an increase in sympathetic nerve activity in response to CIH is expected to result in unregulated release of CA from the adrenal medulla, eventually leading to depletion of CA stores in chromaffin cells. However, by downregulating neurogenic secretion of CA, CIH will prevent such depletion of CA stores. In contrast, by inducing hypoxic sensitivity in adrenal medulla, CIH may facilitate CA secretion only during hypoxic episodes (i.e. regulated secretion). Thus, CIH leads to functional remodelling of adrenal medulla. Chronic intermittent hypoxia has been shown to induce functional plasticity in the respiratory system (Ling *et al.* 2001) and in the carotid body, which manifested as sensory long-term facilitation (Peng *et al.* 2003). The present study on the adrenal medulla provides another elegant example of functional plasticity induced by CIH, which seems to have potential implications in cardiovascular regulation during recurrent periods of apnoea.

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