

Postnatal hypoxemia increases angiotensin II sensitivity and up-regulates AT_{1a} angiotensin receptors in rat carotid body chemoreceptors

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Abstract

In the present study, the effects of postnatal hypoxemia on the AT₁ angiotensin receptor-mediated activities in the rat carotid body were studied. Angiotensin II (Ang II) concentration-dependently increased the chemoreceptor afferent activity in the isolated carotid body. Single- or pauci-fiber recording of the sinus nerve revealed that the afferent response to Ang II was enhanced in the postnatally hypoxic carotid body. To determine whether the increased sensitivity to Ang II is mediated by changes in the functional expression of Ang II receptors in the carotid body chemoreceptors, cytosolic calcium ([Ca²⁺]_i) was measured by spectrofluorimetry in fura-2 acetoxymethyl ester-loaded type I cells dissociated from carotid bodies. Ang II (25–100 nM) concentration-dependently increased [Ca²⁺]_i in the type I cells. The proportion of clusters of type I cells responsive to Ang II was higher in the postnatally hypoxic group than in the normoxic control (89 vs 66%). In addition, the peak [Ca²⁺]_i response to Ang II was enhanced 2- to 3-fold in the postnatally hypoxic

group. The [Ca²⁺]_i response to Ang II was abolished by pretreatment with losartan (1 μM), an AT₁ receptor antagonist, but not by PD-123177 (1 μM), an AT₂ antagonist. Double-labeling immunohistochemistry confirmed that an enhanced immunoreactivity for AT₁ receptor was co-localized to the lobules of type I cells in the hypoxic group. In addition, RT-PCR analysis of subtypes of AT₁ receptors showed an up-regulation of AT_{1a} but a down-regulation of AT_{1b} receptors, indicating a differential regulation of the expression of AT₁ receptor subtypes by postnatal hypoxia in the carotid body. These data suggest that postnatal hypoxemia is associated with an increased sensitivity of peripheral chemoreceptors in response to Ang II and an up-regulation of AT_{1a} receptor-mediated [Ca²⁺]_i activity of the chemoreceptors. This modulation may be important for adaptation of carotid body functions in the hypoxic ventilatory response and in electrolyte and water homeostasis during perinatal and postnatal hypoxia.

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Introduction

Chemoreceptors in the carotid body increase intracellular calcium and afferent nerve discharge in hypoxia and, thus, are essential to the cardio-respiratory performance during hypoxia (for review see Gonzalez (1994)). The carotid body enlarges at high altitude (Arias-Stella & Valcarcel 1976, Honig 1989) or in clinical conditions as observed in patients with chronic hypoxemia (Lack 1978). Of particular interest in this context is chronic hypoxia due to congenital heart disease, cystic fibrosis or sleep apnea, which affects patients of young ages, and whose carotid bodies undergo hypertrophy and hyperplasia (Lack 1978, Lack *et al.* 1985). It is known that the carotid chemoreceptor sensitivity is under maturational change in the perinatal period (Kholwadwala & Donnelly 1992, Wasicko *et al.* 1999). In addition, chronic hypoxia modulates the ventilatory response to acute hypoxia in rat pups

(Eden & Hanson 1987) and the physiological changes are coupled to structural remodeling including increased vasculature and hypertrophy and hyperplasia of the type I cells, i.e. the chemoreceptors in the rat carotid body (Dhillon *et al.* 1984, McGregor *et al.* 1984, Bee *et al.* 1986). However, the molecular mechanisms underlying the modulation of chronic hypoxia during postnatal development and its physiological changes are largely undefined. Therefore, the acclimatization of the carotid body in structure and functions to chronic hypoxia during perinatal and postnatal periods is of great interest in its modulation during early maturation and thus its clinical relevance.

Angiotensin II (Ang II) is a potent vasoconstrictor and it is the major circulating hormone for stimulating aldosterone secretion by the adrenal cortex (Peach 1977). It has been shown that there is an increase in serum levels of non-esterified fatty acid, which inhibits the production of

aldosterone in rabbits exposed to chronic hypoxia from birth (Raff *et al.* 1997). Also, aldosteronogenesis increases in the adrenal glands of neonatal rats during perinatal and postnatal hypoxia, which is opposite to that in the adult (Raff *et al.* 1999b). Hence the salt and water balance and its hormonal regulation are influenced by chronic hypoxia and the effect of hypoxic stress in early maturation is different from that in adults. Ang II has been reported to increase the chemoreceptor afferent activity, presumably via the mediation of the AT₁ angiotensin receptor in the carotid body (Allen 1998, Fung *et al.* 2001). Recently, we have demonstrated that chronic hypoxia up-regulates the expression of Ang II receptors, predominantly the AT₁ receptor in mature rats, which results in enhanced carotid body afferent nerve activity to Ang II stimulation (Leung *et al.* 2000). In view of this, it is more intriguing to study the effects of chronic hypoxia on carotid chemoreceptor function during perinatal and postnatal periods because of (i) the discrepancy between the effects of chronic hypoxia on neonates and adults and (ii) its clinical relevance in such diseases as congenital heart defects, chronic lung disease of prematurity, cystic fibrosis and sleep apnea, which mainly affect babies and young subjects. Accordingly, the present study was aimed at elucidating the cellular and molecular changes underlying the effect of postnatal hypoxemia on the AT₁ receptor-mediated chemoreceptor activity in the rat carotid body.

Materials and Methods

Preparation of animals

For the exposure of rats in normobaric hypoxia, the details of experimental procedures have been described previously (Leung *et al.* 2000, 2001). Briefly, litters of Sprague–Dawley rat pups with the mother were placed in a hypoxic chamber and exposed to inspired oxygen at $10 \pm 0.5\%$ for 4–5 weeks from birth. For normoxic controls, litters were kept in the same room but were supplied with room air. Animal model and experimental protocols were approved by the Committee on the use of Live Animals in Teaching and Research of the University of Hong Kong and the Animal Ethical Committee of the Chinese University of Hong Kong.

Isolation of the carotid body, dissociation of type I cells and spectrofluorimetry

Following deep anesthesia with halothane, rats were decapitated and the carotid bifurcation was excised rapidly. The carotid body was carefully dissected free from the bifurcation in chilled rat Ringer solution oxygenated with 95% O₂ and 5% CO₂. The carotid body was then incubated in a tissue bath with collagenase (0.06%) and protease (0.02%) in oxygenated Ringer solution for 30 min at 35 ± 1 °C (Fung *et al.* 2001).

Following enzymatic treatment, the carotid body cells were dispersed by gently triturating with glass pipettes (Fung *et al.* 2001). Cells were incubated in 5 µM fura-2 acetoxymethyl ester (fura-2) (fura-2AM; Molecular Probes, Eugene, OR, USA) for 30 min at room temperature. The cells were then centrifuged at 200 g for 5 min and prepared for spectrofluorimetric measurement of cytosolic calcium ([Ca²⁺]_i) (Biscoe *et al.* 1989, Wasicko *et al.* 1999). Type I cells in clusters of 8–20 cells were studied; the morphological criteria for their confirmation as type I cells was described in a previous report (Donnelly & Kholwadwala 1992). Freshly dissociated type I cells tended to occur in clumps, and were birefringent with diameters of 7–10 µm. These cells could consistently respond to hypoxic stimuli several hours following the dissociation.

[Ca²⁺]_i was measured in fura-2-loaded type I cells freshly dissociated from rat carotid bodies as described in previous studies (Biscoe *et al.* 1989, Fung *et al.* 2001). The cells were seeded on a cover slip placed on the stage of an inverted microscope. The microscope was coupled with a dual-wavelength excitation spectrofluorimeter (Photon Technology International, Lawrenceville, NJ, USA). The cells were perfused with Hepes buffer at 0.5 ml/min at room temperature (~22 °C). The fluorescence intensity of the background was measured and subtracted from the signals. Fluorescent signals were obtained at 340 and 380 nm excitation wavelengths. The ratio of the fluorescence intensity (340 nm/380 nm) was used to estimate [Ca²⁺]_i in the type I cells. [Ca²⁺]_i was calculated by using the equation: $[Ca^{2+}]_i = K_d [(R_o - R_{min}) / (R_{max} - R_o)] \beta$, where R_o is the fluorescence ratio, R_{min} is the fluorescence ratio at zero Ca²⁺, R_{max} is the fluorescence ratio at saturated Ca²⁺, K_d is the dissociation constant for fura-2 (224 nM) and β is the ratio of 380 nm fluorescence intensity at zero Ca²⁺ to 380 nm fluorescence intensity at saturated Ca²⁺.

In vivo electrophysiology

The carotid body was held in the recording chamber at 35 ± 1 °C, and perfused (2–3 ml/min) with oxygenated Ringer solution and the sinus nerve was recorded for single- or pauci-fiber activities with suction electrodes. The signal was amplified, filtered, monitored, digitized and stored for analysis. Ang II (0.1–100 nM) was perfused for 3 min for the concentration-dependent response. Acute hypoxia was induced by perfusion of rat Ringer solution gassed with 95% N₂ and 5% CO₂ to confirm the chemosensitivity of the afferent fibers.

Experimental paradigm

The [Ca²⁺]_i response to Ang II was determined at 25, 50 and 100 nM. Ang II was administered directly into the 0.5 ml chamber with a fine pipette without disrupting the

fluid in the chamber. The antagonist for AT₁, losartan (Merck & Co., Inc., NJ, USA) (1 μM), or for AT₂, PD-123177 (Parke-Davis Pharmaceutical Research, Detroit, MI, USA) (1 μM), was perfused for 3 min before Ang II treatment. At the end of the experiment, acute hypoxia was induced by NaCN (2 mM, in bolus) to confirm the chemosensitivity of the type I cells.

RT-PCR

The procedures have been described previously (Leung *et al.* 2000). Total RNA was subjected to first-strand cDNA synthesis using random hexamer primers and Superscript II transcriptase (GIBCO-BRL, Grand Island, NY, USA) in a final volume of 20 μl. After incubation at 42 °C for 1 h, the reaction mixture was treated with RNase H before proceeding to PCR analysis. The final mixture (2 μl) was directly used for PCR amplification. mRNAs of Ang II receptor subtypes, namely AT_{1a} and AT_{1b}, were detected with primers as employed previously. All RNA was tested for freedom from DNA contamination by RT-PCR without addition of reverse transcriptase. After appropriate validation for the semi-quantitative RT-PCR as reported previously (Chan *et al.* 2000, Leung *et al.* 2000), all samples were analyzed for both Ang II receptor subtype and β-actin genes in the logarithmic phase of the amplification reactions. The PCR conditions were 30 cycles of: denaturing, 94 °C, 1 min; annealing, 58 °C for AT_{1a} and 62 °C for AT_{1b}, 1 min; and elongating, 72 °C, 2 min. The amplified mixture (10 μl) was finally separated on 2% agarose gel electrophoresis and the amplified DNA bands were detected using ethidium bromide staining. The bands were then quantified with an image analyzer (Image Quant; Molecular Dynamics, Sunnyvale, CA, USA).

Immunohistochemical localization of AT₁ receptor

The carotid bifurcations from normoxic and hypoxic Sprague-Dawley rats (*n*=6) were removed bilaterally from the head, rinsed in PBS and frozen in isopentane. Cryosections (8 μm) were fixed with freshly prepared paraformaldehyde (4%) for 30 min. The sections were then processed for indirect immunofluorescent double staining. Sections were incubated overnight at 4 °C with anti-AT₁ receptor serum (Santa Cruz Biotech. Inc., Santa Cruz, CA, USA), diluted to 0.2 μg/ml and with anti-tyrosine hydroxylase (TH) serum (Chemicon International Inc., Temecula, CA, USA), diluted to 1:500 overnight at 4 °C. After thorough washing with PBS, the primary antibodies were detected using anti-rabbit labeled with rhodamine for AT₁ receptor and anti-sheep labeled with aminomethylcoumarin for TH (Chemicon International) for 2 h at room temperature. Positive immunoreactivity for AT₁ receptor (red) and for TH (blue) was examined with a fluorescent microscope equipped with a DC 200 digital

camera (Leica Microsystems Ltd, Bannockburn, IL, USA). The following controls were used: (i) substitution of primary antibodies with buffer; (ii) incubation with rabbit non-immune serum; and (iii) liquid phase preadsorption of AT₁ receptor with excess blocking peptide (Santa Cruz Biotech.).

Materials and pharmacological agents

The rat Ringer solution contained (mM): NaCl 125, KCl 3.1, NaHCO₃ 26, NaH₂PO₄ 1.25, MgSO₄ 1.3, CaCl₂ 2.4, D-dextrose 10 (pH 7.35–7.40). The Hepes-Ringer solution contained (mM): NaCl 140, KCl 3, NaH₂PO₄ 1.25, MgCl₂ 1, CaCl₂ 1, Hepes 10, D-dextrose 25 (pH 7.35–7.40). Antagonists for Ang II receptors were dissolved in Hepes-Ringer solution. The concentrations of Ang II and NaCN were 5 μM and 1 M respectively for the injection. Fura-2AM was dissolved in DMSO in 1 mM stock. Chemicals were purchased from Sigma Chemical Co., St Louis, MO, USA, unless otherwise stated.

Data analysis

For [Ca²⁺]_i, the resting and peak values of the fluorescence ratio of 340/380 nm or calibrated [Ca²⁺]_i (nM) of the responses during drug treatment were calculated. Values were normalized to percent of control if needed and are presented as means ± S.E.M. Statistical comparisons were made with the paired *t*-test or the non-parametric Wilcoxon signed rank test for comparing pre- and post-treatment. ANOVAs with *post hoc* tests (Dunnett *t*-test) were used for multiple comparisons of values in drug studies among groups with different doses. Differences were considered significant at *P*<0.05. For RT-PCR, mRNA expression was normalized as percent of control of β-actin. Results are expressed as means ± S.E.M. for the normoxic controls and the chronically hypoxic group. Differences were compared using an unpaired *t*-test (considered significant at *P*<0.05).

Results

Ang II increases afferent activities of the carotid body *in vitro*

In all carotid bodies (*n*=16) tested, Ang II increased the sinus nerve activity. There were no differences (*P*=0.86, unpaired *t*-test) in the resting discharge rate between the normoxic (0.15 ± 0.03 Hz, *n*=8) and hypoxic group (0.16 ± 0.02 Hz, *n*=8). An example is shown in Fig. 1 of Ang II exciting the afferent nerve activity of a carotid body isolated from postnatal chronic hypoxic (CH) rats. The perfusion of Ang II (100 nM) increased the basal discharge rate of a unitary activity (Fig. 1A–D). In addition, Ang II (0.1–100 nM) concentration-dependently increased afferent discharge of the carotid bodies in normoxic and CH rats. Furthermore, the Ang II response in

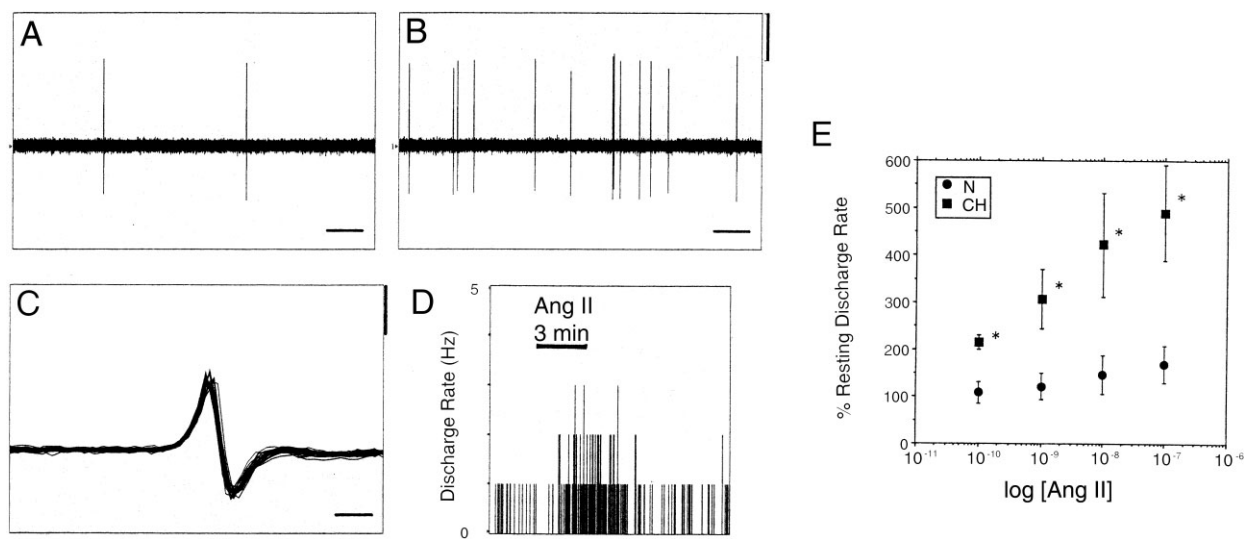


Figure 1 Ang II-increased afferent nerve activities of the carotid body *in vitro*. The carotid body was from a 30-day-old rat with chronic hypoxia from birth and had a low basal activity (A). As shown in (B), the unitary activity increased following the perfusion of Ang II (100 nM) for 3 min. The waveform of the unitary activity is shown in (C) by overlapping plots of 30 spikes. The discharge rate is shown in (D) and the perfusion of Ang II increased this rate. The dose-dependent curves of the excitatory effect of Ang II on the nerve discharge in normoxic (N, $n=8$) and chronic hypoxic (CH, $n=8$) groups are shown in (E). Data are means \pm S.E.M. Note that the concentration of Ang II is plotted logarithmically and that the Ang II response was greater in the CH group than in the normoxic controls. Calibration bars for (A and B) are 2 s; for (C) 0.44 ms; for (D) 3 min. * $P<0.05$ compared with the N group.

the CH group was greater than in the normoxic group. On average, the response to Ang II (100 nM) was $490 \pm 101.8\%$ ($n=8$) in the carotid body from CH rats compared with $169 \pm 38.9\%$ ($n=8$) in the normoxic rats (Fig. 1E). Hence, the dose–response curve for the CH group was upward and leftward shifted (Fig. 1E), suggesting that receptor binding to the Ang II is increased in the CH carotid body.

$[Ca^{2+}]_i$ response to Ang II

Of 47 clusters of type I cells examined in the normoxic group, 31 (66%) responded to Ang II (25–100 nM). In the CH group, 48 of 54 clusters (89%) of type I cells responded to Ang II (25–100 nM). Ang II elevated $[Ca^{2+}]_i$ in all of the responsive cells (Fig. 2A and B). The peak $[Ca^{2+}]_i$ response was reached shortly following Ang II stimulation and $[Ca^{2+}]_i$ gradually returned to the resting level within 3 min (Fig. 2A and B). The $[Ca^{2+}]_i$ response to Ang II was significantly greater in the CH than in the normoxic group (Fig. 2C). On average, $[Ca^{2+}]_i$ increase in response to Ang II (100 nM) was 20.8 ± 3.2 nM ($n=20$) and 58.3 ± 11.7 nM ($n=20$) in normoxic and CH groups respectively. In addition, $[Ca^{2+}]_i$ increase in response to Ang II was concentration-dependent in both groups (Fig. 2C). There was no significant difference in the resting $[Ca^{2+}]_i$ between the normoxic (195.1 ± 27.5 nM, $n=31$) and hypoxic groups (211.0 ± 35.9 nM, $n=48$) ($P=0.13$, unpaired *t*-test).

Blockade of Ang II receptor subtypes

Losartan (1 μ M) treatment largely abolished the $[Ca^{2+}]_i$ response to Ang II in the type I cells from the CH carotid bodies (Fig. 3A and C) and the treatment *per se* did not change the resting $[Ca^{2+}]_i$ ($P=0.36$, paired *t*-test, $n=6$). On average, the Ang II-induced $[Ca^{2+}]_i$ peak changes pre- and post-treatment were significantly different ($P=0.02$, paired *t*-test, $n=6$) (Fig. 3C). However, PD-123177 treatment did not cause any significant difference in the peak $[Ca^{2+}]_i$ response to Ang II ($P=0.46$, $n=6$) (Fig. 3B and C) and the treatment *per se* did not change $[Ca^{2+}]_i$ ($P=0.36$, paired *t*-test, $n=6$). Moreover, vehicle treatment did not change the $[Ca^{2+}]_i$ response to Ang II stimulation ($P=0.2$, Wilcoxon signed rank test, $n=3$).

Transcriptional expression of the AT_1 receptor subtypes

The regulation of mRNA expression of AT_1 receptors at both AT_{1a} and AT_{1b} subtypes in the carotid body was investigated using semi-quantitative RT-PCR. There was a marked and differential effect of chronic hypoxia on the expression of AT_{1a} and AT_{1b} subtypes. Postnatal hypoxia elicited a significant increase in the mRNA expression for AT_{1a} subtype when compared with its control (Fig. 4). The relative change of expression of the AT_{1a} subtype was about 4-fold, as demonstrated by RT-PCR and image analysis. However, postnatal hypoxia caused a decrease in mRNA expression for AT_{1b} subtype when compared with its control (Fig. 5).

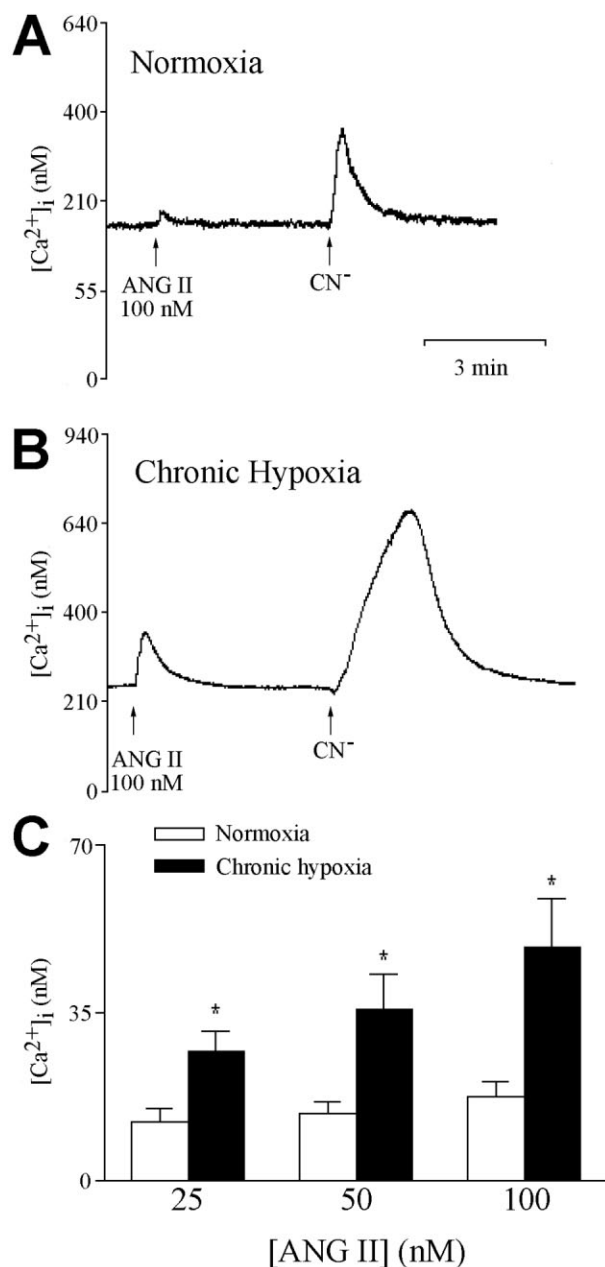


Figure 2 Ang II-elevated cytosolic calcium ($[Ca^{2+}]_i$) in dissociated type I cells of the rat carotid body from normoxic control (A) and postnatally CH (B) groups. The records were made in a cluster of 8–10 cells loaded with fura-2. Note the transient increase of $[Ca^{2+}]_i$ following the injection of Ang II (100 nM) into the bath. In addition, histotoxic hypoxia with NaCN (CN^- , 2 mM) increased $[Ca^{2+}]_i$ of the dissociated type I cells. The Ang II-induced $[Ca^{2+}]_i$ was greater in the CH group (B) than in the normoxic control (A). (C) Summary showing that, on average, Ang II-induced $[Ca^{2+}]_i$ in the type I cells from normoxic controls was less than that in the CH group. Data are means \pm S.E.M. The number of cell preparations (in 25, 50, 100 nM) are 11, 10 and 20, and 7, 6 and 20 in the normoxic and hypoxic groups respectively. * $P < 0.05$ compared with the normoxic group.

Double labeling of AT_1 receptors and TH

To further demonstrate the effect of postnatal hypoxia on the localization of AT_1 receptor in the type I glomus cells, immunohistochemistry coupled with a double-labeling technique was employed. Results showed that distinct immunostaining of AT_1 receptor was predominantly localized to the cell clusters of glomus cells (Fig. 6), which was validated by the presence of TH as an immunohistochemical marker for parenchymal type I glomus cells (Kameda *et al.* 1990). Detailed examination showed that stronger immunostaining of AT_1 receptor was detected in the carotid body in postnatal hypoxia (Fig. 6B) when compared with that in normoxic controls (Fig. 6A). In addition, intense immunostaining of TH was specifically observed in the cytoplasm of type I glomus cells. More intense immunostaining was detected in hypoxic carotid bodies (Fig. 6D) when compared with that in normoxic carotid bodies (Fig. 6C). Immunoreactivity for AT_1 receptor was localized predominantly to the cell clusters of glomus cells as demonstrated by an overlay of AT_1 receptor and TH immunostaining, either in normoxic (Fig. 6E) or in hypoxic (Fig. 6F) carotid body. Specificity of the immunostaining was demonstrated by the negative control experiments when specific antibody was preadsorbed in excess with its AT_1 receptor antigen either in normoxic (Fig. 6G) or in hypoxic (Fig. 6H) carotid body.

Discussion

This is the first study to demonstrate (i) that chronic hypoxia from birth increases chemoreceptor sensitivity to Ang II in the rat carotid body, and (ii) that the augmentation of the Ang II sensitivity is mediated by a differential modulation of the expression of AT_1 receptor subtypes in the chemoreceptor of the carotid body. Hence, we have shown that postnatal hypoxia enhanced: (i) the nerve afferent response to Ang II in the carotid body; (ii) the $[Ca^{2+}]_i$ response to Ang II in type I cells; and (iii) AT_1 receptors localized to the lobules of type I glomus cells. In addition, the $[Ca^{2+}]_i$ response to Ang II was blocked by an antagonist for AT_1 subtype but not by an antagonist for AT_2 subtype, confirming that Ang II binding to the AT_1 receptor stimulates the intracellular signaling pathway and elevates $[Ca^{2+}]_i$ in the type I cells. Moreover, the gene transcript of AT_{1a} subtype was increased whereas AT_{1b} was decreased in the carotid body by hypoxia. Taken together, these results support the hypothesis that postnatal hypoxia increases AT_{1a} receptors expressed in the chemoreceptor of the carotid body and this may be responsible for the enhancement of the Ang II sensitivity.

Previous studies have shown that chronic hypoxia reduces growth rate in rats, which may be due to an impairment of muscle and fat growth as well as a reduction

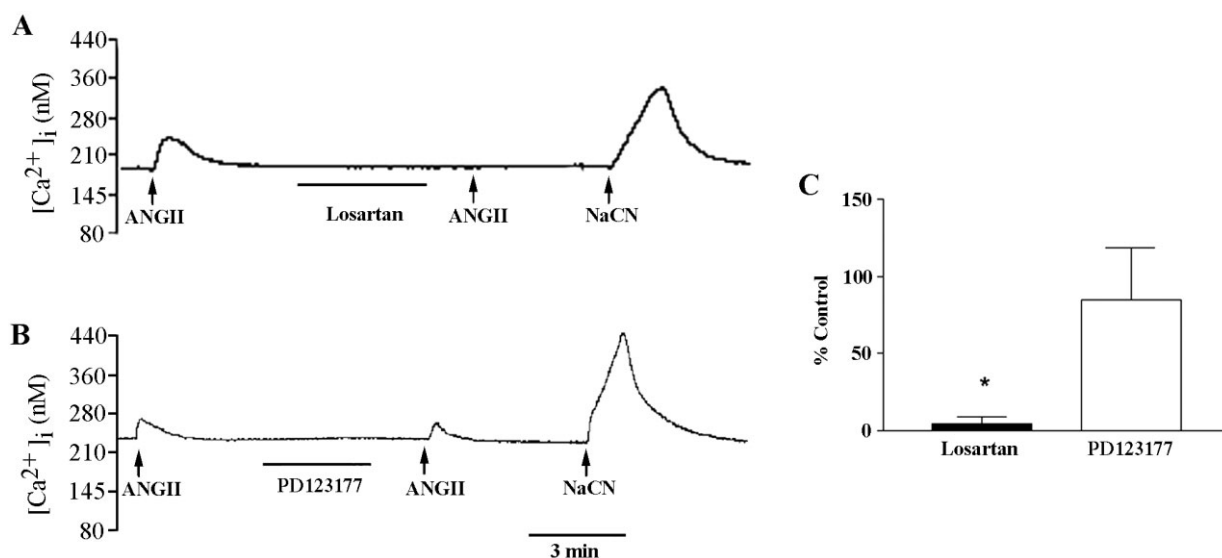


Figure 3 Effects of losartan and PD-123177 on the Ang II-induced $[Ca^{2+}]_i$ change in type I cells from postnatally hypoxic rats. (A) Blockade of AT₁ subtypes of Ang II receptors with losartan pretreatment largely abolished the $[Ca^{2+}]_i$ response to Ang II (100 nM, arrows) in a cluster of type I cells. (B) However, blockade of AT₂ subtypes of Ang II receptors with PD-123177 did not attenuate the $[Ca^{2+}]_i$ response to Ang II (100 nM, arrows) in another cluster of type I cells. The effects of losartan and PD-123177 on the Ang II-induced $[Ca^{2+}]_i$ change is summarized in (C). Data are means \pm S.E.M. ($n=6$) * $P<0.05$ relative to the Ang II-induced $[Ca^{2+}]_i$ response of the corresponding pretreatment group.

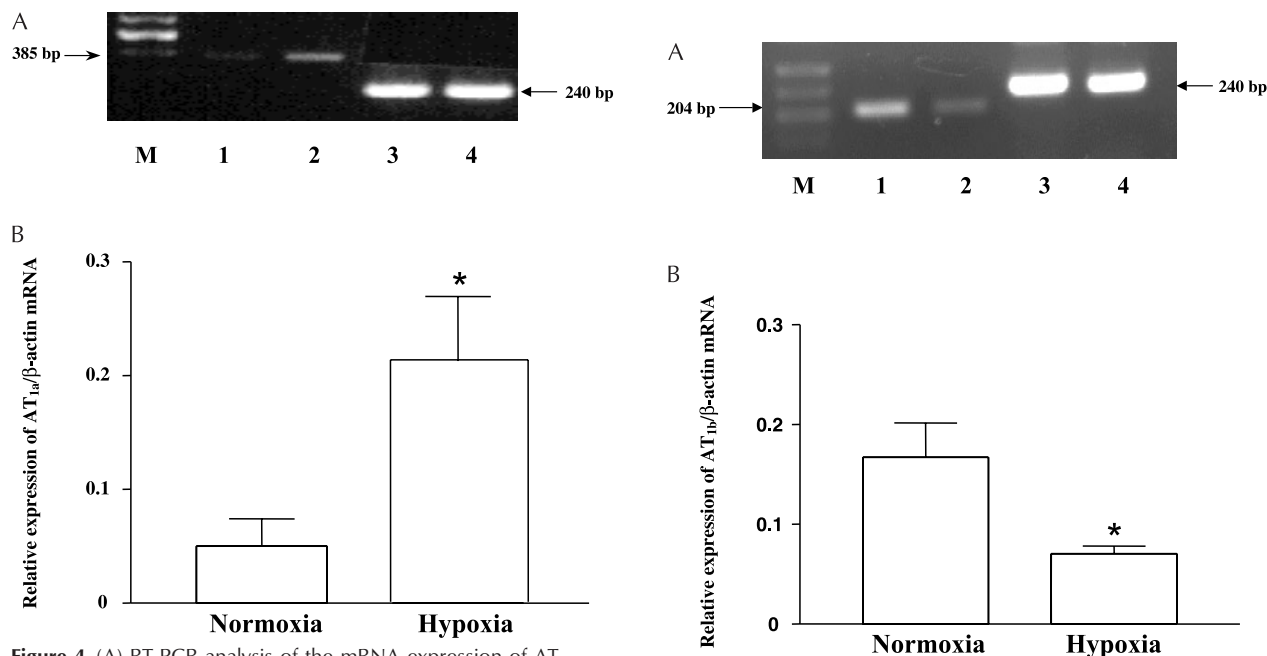


Figure 4 (A) RT-PCR analysis of the mRNA expression of AT_{1a} receptor in the rat carotid body in postnatal hypoxia. Lane M, DNA marker; lane 1, AT_{1a} receptor expression in normoxic (control) carotid bodies; lane 2, AT_{1a} receptor expression in hypoxic carotid bodies; lane 3, β -actin expression in the control carotid bodies; lane 4, β -actin expression in hypoxic carotid bodies. The arrows indicate the expected size of amplified products from AT_{1a} receptor (385 bp) and β -actin (240 bp). (B) The relative expression of AT_{1a} receptor/ β -actin mRNA in the normoxic and chronically hypoxic group of carotid bodies. Data are means \pm S.E.M. ($n=6$ /group); * $P<0.05$.

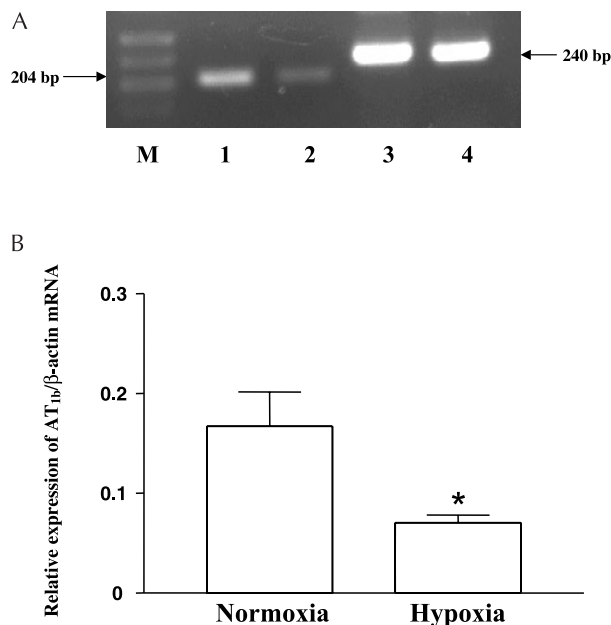


Figure 5 (A) RT-PCR analysis of the mRNA expression of AT_{1b} receptor in the rat carotid body in postnatal hypoxia. Lane M, DNA marker; lane 1, AT_{1b} receptor expression in control carotid bodies; lane 2, AT_{1b} receptor expression in hypoxic carotid bodies; lane 3, β -actin expression in control carotid bodies; lane 4, β -actin expression in the hypoxic carotid bodies. The arrows indicate the expected size of amplified products from AT_{1b} receptor (204 bp) and β -actin (240 bp). (B) The relative expression of AT_{1b} receptor/ β -actin mRNA. Data are means \pm S.E.M. ($n=6$ /group); * $P<0.05$.

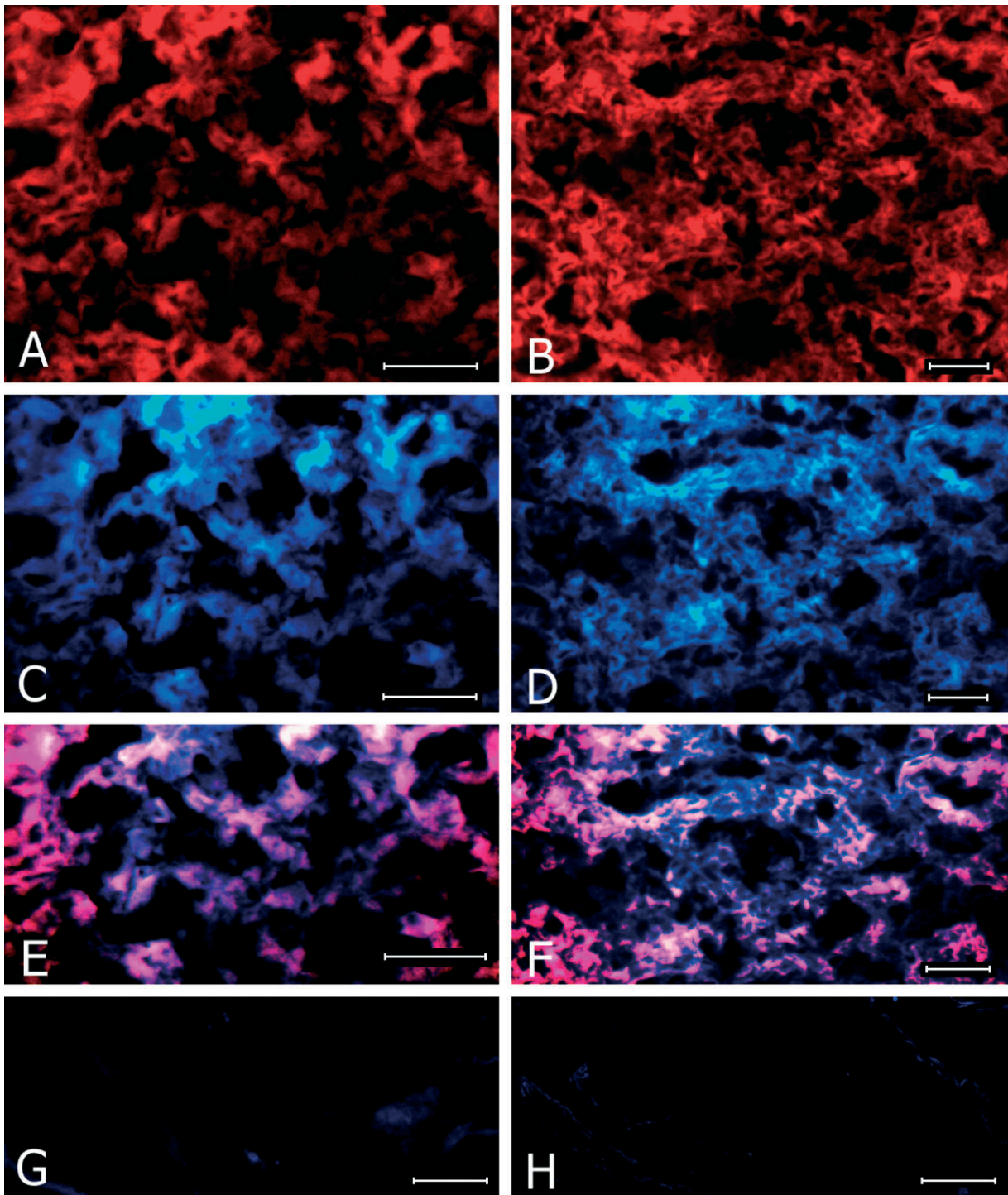


Figure 6 Immunohistochemical localization of AT_1 receptor and tyrosine hydroxylase (TH) in carotid body of postnatal hypoxia. Distinct immunoreactivity for AT_1 receptor (red) was localized to the perinuclear cytoplasm of glomus cells in the carotid body from normoxia (A) and hypoxia (B). Intense immunoreactivity for TH (blue) was consistently and predominantly localized to glomus cells from normoxic (C) and in hypoxic (D) carotid body. Immunoreactivity for AT_1 receptor was co-localized predominantly with that for TH in the cell clusters of glomus cells (purple) as demonstrated by an overlay of AT_1 receptor and TH immunostaining, either in normoxic (E) or in hypoxic (F) carotid body. No immunostaining was observed in preadsorption of primary antibody with excess of AT_1 receptor antigen either in normoxic (G) or in hypoxia rats (H). The scale bars represent 20 μm (A–F) and 50 μm (G & H).

in food consumption (Bigard *et al.* 1996, Raff *et al.* 1999a, Raff *et al.* 2001). In this study, we also observed that the body weight of rats exposed to postnatal hypoxia (52.3 ± 2.4 g, $n=8$) was significantly less ($P<0.0001$, unpaired *t*-test) than that of the normoxic control (91.3 ± 4.2 g, $n=8$). Despite the decreased growth rate, we found that the afferent activity and the $[Ca^{2+}]_i$ response to Ang II stimulation were consistently augmented in the hypoxic rats, suggesting a specific effect of hypoxia on the function of the carotid body. Apparently, the augmentation of Ang II sensitivity is parallel to our previous findings in adult rats exposed to chronic hypoxia (Leung *et al.* 2000) and the change is also mediated by the AT₁ receptors. Yet the molecular modulation underlying the effect of chronic hypoxia in the rat pups may be different from that in the adults. Indeed, the RT-PCR data demonstrated unequivocally an up-regulation of AT_{1a} but a down-regulation of AT_{1b} subtypes, indicating a differential expression of AT₁ receptor subtypes during postnatal hypoxia. In adults, chronic hypoxia increases the transcriptional expression of both AT_{1a} and AT_{1b} receptor subtypes in the carotid body (Leung *et al.* 2000). In this context, it has been shown that mRNA expression for AT₁ receptor subtypes is selectively up-regulated by chronic hypoxia in tissues such as the pancreas. Such differential changes of AT₁ receptor subtypes may be responsible for finely regulating the physiology and adaptation of the tissues and organs during chronic hypoxia (Leung & Carlsson 2001). Also, as mentioned in the Introduction, the effect of hypoxia on the production of aldosterone is quite distinct between rat pups and the adults. Hence the molecular regulation of the hypoxic effects is likely to be affected by the maturation.

Our data indicate that the increased chemoreceptor afferent response to Ang II during perinatal and postnatal hypoxia may be due to an up-regulation of AT_{1a} receptor subtype, presumably expressed in type I cells of the carotid body. In fact, results of the double-immunostaining study confirmed an increased AT₁ immunoreactivity co-localized with the TH-containing type I cells and this could represent the up-regulation of the AT_{1a} subtypes in the chemoreceptors. Thus the increased amount of AT_{1a} receptors in type I cells may enhance the intracellular calcium response to Ang II, although at this moment we cannot exclude the potential role played by AT_{1b} subtypes in the carotid body on the Ang II sensitivity and its enhancement during postnatal hypoxia. If so, we may speculate either a negative control pathway mediated by the activation of AT_{1b} receptors or a minor role of the receptor in the $[Ca^{2+}]_i$ response to Ang II. Nevertheless, the significance of changes in AT₁ receptor subtypes in the carotid body during postnatal hypoxia has yet to be elucidated.

The present study clearly showed the effects of postnatal hypoxia on the functional expression of AT₁ receptor-mediated $[Ca^{2+}]_i$ activity in the chemoreceptor. As

proposed in renal and cardiac cells, Ang II binding to AT₁ receptor could stimulate G-protein activity and the phosphatidylinositol/phospholipase C pathway for the release of intracellular calcium store which raises $[Ca^{2+}]_i$ (Abdellatif *et al.* 1991, Matsusaka & Ichikawa. 1997). We have shown that $[Ca^{2+}]_i$ increased following the activation of AT₁ receptors in the type I cells of the rat carotid body (Fung *et al.* 2001). Elevation of intracellular calcium may be coupled to the vesicular secretion of catecholamine for the chemotransduction (Gonzalez *et al.* 1994) and is associated with acute hypoxia or histotoxic hypoxia in dissociated type I cells from rabbits and rats (Biscoe *et al.* 1989, Biscoe & Duchon 1990, Donnelly & Kholwadwala 1992, Bright *et al.* 1996, Wasicko *et al.* 1999).

Perinatal and postnatal hypoxia are of importance in high altitude physiology and in clinical conditions such as congenital heart defects, chronic lung disease of prematurity, cystic fibrosis and sleep apnea, which mainly affect babies and young subjects (Fletcher 2001, Bee & Howard 1993). Consistent with previous reports showing the enlargement of the carotid body during chronic hypoxia in adults, postnatal hypoxia increased the volume of the carotid body 2- to 4-fold in the rat pups. It is possible that the augmentation of Ang II sensitivity in the chemoreceptors may play a role in the hypertrophy and hyperplasia of the type I cells. In addition, postnatal hypoxia attenuates the ventilatory response to hypoxia (Eden & Hanson 1987) and the chemosensitivity of the carotid body may be determined by the balance between the excitatory and inhibitory components in the carotid body (Bisgard 2000). Thus the excitatory effect of Ang II on the chemoreceptor may increase the chemosensitivity of the carotid body and counteract the blunting effect of chronic hypoxia, although the detail of the mechanism underlying the 'blunting' remains unknown. Finally, chronic hypoxia changes the sodium and water content of the blood (Honig 1989) and stimulates the renin-angiotensin system (Gould & Goodman 1970). Although the plasma Ang II concentration initially increases but returns to resting level (Zakheim *et al.* 1976) and the plasma renin activity remains unchanged during chronic hypoxia (Jain *et al.* 1990), the enhancement of the chemoreceptor sensitivity to Ang II may provide a mechanism by which the carotid chemoreceptor activity maintains the renal sympathetic activity which facilitates sodium reabsorption and water intake.

In summary, our results suggest that postnatal hypoxia is associated with an enhanced sensitivity of chemoreceptor activities to Ang II via an AT₁ receptor-mediated pathway. Also, postnatal hypoxia up-regulated AT_{1a} but down-regulated AT_{1b} receptor subtypes in the type I cells of the carotid body, suggesting an involvement of AT_{1a} subtype in the functional change. This modulation may be important for the adaptation of carotid body functions in the hypoxic ventilatory response and in electrolyte and water homeostasis during perinatal and postnatal hypoxia.

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