Chronic hypoxia upregulates the expression and function of AT$_1$ receptor in rat carotid body

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Abstract

In the present study, the effects of chronic hypoxia on the expression and localization of angiotensin II (Ang II) receptors are investigated by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) and by immunohistochemistry. The effect of chronic hypoxia on the carotid body chemoreceptor activity was also examined by in vitro electrophysiology. Results from RT-PCR revealed that chronic hypoxia exhibited differential effects on the gene expression of Ang II receptors, namely AT$_1$ and AT$_2$, in the carotid body. The mRNA expression for subtypes of the AT$_1$ receptor, AT$_{1a}$ and AT$_{1b}$, was significantly increased in the carotid body with chronic hypoxia. To further investigate the localization of the AT$_1$ receptor, an immunohistochemical study was performed. The results showed that AT$_1$ receptor immunoreactivity was found in lobules of glomus cells in the carotid body and the immunoreactivity was more intense in chronic hypoxia than in normoxic controls. In vitro electrophysiological studies consistently demonstrated that chronic hypoxia enhanced the AT$_1$ receptor-mediated excitation of carotid body chemoreceptor activity. These data suggest that chronic hypoxia upregulates the transcriptional and post-transcriptional expression of AT$_1$ receptors in the rat carotid body. The upregulation of the expression also enhances AT$_1$ receptor-mediated excitation of the carotid body afferent activity. This might be important in the modulation of cardiorespiratory functions as well as fluid and electrolyte homeostasis during chronic hypoxia.

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Introduction

The carotid body is located in the bifurcation of the carotid arteries and plays a central role in cardiorespiratory responses to physiological stimuli such as changes in arterial blood gases, pH, or osmolality. In hypoxia, chemoreceptors in the carotid body increase the afferent activity of the carotid sinus nerves, which activates brainstem for the elevation of respiratory drive (Gonzalez et al. 1994). The stimulation of carotid body chemoreceptors also reflexly increases cardiac output, sympathetic flow and elevates circulating vasoactive hormones such as norepinephrine and angiotensin II (Ang II) for enhancing cardiovascular performance (Marshall 1994).

Ang II is a physiologically active component of the renin–angiotensin system (RAS), which plays an important role in regulating the blood pressure and fluid homeostasis (Peach 1977). It has been known that both systemic and tissue RAS are activated by hypoxia. In the kidney, RAS was shown to be upregulated by chronic hypoxia (Neylon et al. 1997, Fletcher et al. 1999). In addition, it has recently been demonstrated that chronic hypoxia activates the expression of local RAS component genes in the pancreas (Chan et al. 2000). Interestingly, peripheral infusion of Ang II stimulates cardiorespiratory functions (Ohtake & Jennings et al. 1993) and the plasma Ang II level increases during hypoxia (Zakheim et al. 1976). Recently, Ang II has been demonstrated to increase the carotid body afferent activity, presumably via the mediation of the AT$_1$ receptor (Allen 1998). Thus activation of Ang II receptors in the carotid body may stimulate cardiorespiratory functions and play a role in the fluid and electrolyte homeostasis during hypoxia. In fact, carotid body functions are modulated by chronic hypoxia. For example, the carotid body releases catecholamines during hypoxia and this is believed to be an important step for the chemotransduction (Donnelly 1993). In chronic hypoxia, the production of catecholamines elevates in the carotid body and this is associated with an increase in the amount and activity of tyrosine hydroxylase, as demonstrated in rat (Gonzalez et al. 1979, Hanbauer et al. 1981, Czyzyk-Krzeska et al. 1992) and in humans (Lack et al. 1985). Moreover, carotid afferent activities are known to play a role in the natriuresis and diuresis during chronic hypoxia, although the underlying mechanism is not clear (Honig 1989).
Provided that Ang II excites carotid body afferent activity, the carotid body response to Ang II may be modulated by chronic hypoxia and this could enhance the afferent activity, which potentiate the cardiorespiratory response as well as the natriuretic and diuretic response during chronic hypoxia. Accordingly, the present study is aimed at elaborating the regulation of chronic hypoxia on the expression and localization of Ang II receptors and at elucidating its effect on the chemoreceptor activity of the carotid body.

**Materials and Methods**

**Animals and isolation of carotid body**

Adult Sprague–Dawley (SD) rats of either sex weighing about 70 g at age 28 days were employed in the present study. The animals were bred in the Laboratory Animal Services Centre, the Chinese University of Hong Kong. The animals were supplied with food and water and allowed to feed ad libitum. Animal model and experimental procedure have been approved by the Animal Ethical Committee of the Chinese University of Hong Kong, Hong Kong. Following deep anaesthesia with halothane, SD rats were decapitated and the carotid body was dissected out from the bifurcation and the superior sympathetic ganglion in PBS, pH 7.4.

**Materials and pharmacological agents**

The rat Ringer solution contained (mM): NaCl 125, KCl 3.1, NaH₂PO₄ 1.25, MgSO₄ 1.3, CaCl₂ 2.4, d-Dextrose 10. Pharmacological agents or drugs were obtained from Sigma, RBI and Dupont Pharmaceutical.

**Chronic hypoxic exposure**

For the exposure of rats in chronic hypoxia, SD rats aged 28 days were raised inside an acrylic chamber filled with 10% ± 0.5% oxygen for isotopic hypoxia for 4 weeks, as reported previously in our laboratory (Chan et al. 2000). Briefly, the oxygen level was monitored continuously by an oxygen analyser and pure nitrogen was mixed with room air. The humidity and carbon dioxide level were maintained by desiccators and by soda lime placed inside the chamber. For normoxic controls, time- and age-matched rats were kept in the same housing and allowed to breathe room air.

**Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) of Ang II receptors**

Total RNA was isolated from carotid body and adrenal gland which was employed as a positive tissue for the expression of Ang II receptors, according to the acid guanidinium thiocyanate–phenol–chloroform protocol (Chomczynski & Sacchi 1987). Briefly, tissues were homogenized in 4 M guanidinium thiocyanate solution. The extracted total RNA was studied by gel electrophoresis and quantified by spectrophotometry. Total RNA (10 µg) 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 polymerase chain reaction (PCR) analysis.

The final mixture (2 µl) was directly used for PCR amplification. mRNAs of Ang II receptor subtypes, namely AT₁a, AT₁b and AT₂ were detected with primers as employed previously (Leung et al. 1998, 1999a). All RNA was tested to be free of 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), all samples were analysed 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 AT₁a and AT₂ and 62 °C for AT₁b, 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 image analyser (Molecular Dynamics Image Quant, Sunnyvale, CA, USA).

**Immunohistochemical localization of AT₁ receptor**

The carotid bifurcation from normoxic and hypoxic SD rats (n=8) were removed bilaterally from the head, rinsed in PBS and frozen in iso-pentane. Consecutive cryosections (8–10 µm) were fixed with freshly prepared paraformaldehyde (4%) for 30 min. The sections were then processed for indirect immunoperoxidase staining as described previously (Leung et al. 1999a,b). Briefly, 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, or with anti-tyrosine hydroxylase serum (Chemicon International Inc., CA, USA), diluted to 1:2000 overnight at 4 °C. The primary antibody was detected using an avidin–biotin conjugate kit (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA, USA) according to the manufacturer’s instructions. Positive immunoreactivity was visualized by reacting sections with Vector VIP substrate kit (Vector Laboratories). Sections were slightly counterstained with haematoxylin, dehydrated and mounted. The following controls were used: (i) substitution of primary antibodies with buffer; (ii) incubation with rabbit non-immune serum; (iii) liquid phase pre-adsorption of AT₁ receptor with excess blocking peptide (Santa Cruz Biotech. Inc).
In vitro electrophysiological study of AT_1 receptors in chronically hypoxic carotid body

The isolated carotid body was incubated in collagenase and protease for 30 min at 35 °C (Kholwadwala & Donnelly 1992). It was then held in the recording chamber at 35 ± 1 °C, and perfused (2–3 ml/min) with oxygenated Ringer and the sinus nerve was recorded for single- or pauci-fiber activities with suction electrodes. The signal was amplified, filtered, monitored and digitized for offline analysis. Ang II (0·1–100 nM) was perfused for 3 min for the dose-dependent response. For the blockade of the AT_1 receptor losartan (10 µM), was perfused for 10 min before the Ang II (100 nM) treatment. Acute hypoxia was induced by perfusion of rat Ringer gassed with (95% N_2 and 5% CO_2) to confirm the chemosensitivity of the afferent fibers.

Data analysis and statistics

For RT-PCR, the Ang II receptor expression is normalized in % control of the β-actin expression. Results were expressed as the means ± s.e.m.s for the normoxic controls and the chronically hypoxic group. Statistical comparisons were made by ANOVA and differences between individual mean values were compared using unpaired Student’s t-test. Values of P<0·05 were considered statistically significant. For in vitro electrophysiology, we compared the discharge rate (spike/s, average over 1–3 min) before, during and after the drug treatment. Values were normalized in % control of resting activity and were presented as means ± s.e.m.s. Statistical comparisons between the response to Ang II in normoxic and chronically hypoxic group were made by ANOVA with post hoc tests. The same analysis was used for comparison in drugs studies among groups. Differences were considered significant at P<0·05.

Results

Effect of chronic hypoxia on the expression of AT_1 receptors

The mRNA expression of AT_1 receptors at both AT_1a and AT_1b subtypes in the carotid body was investigated using RT-PCR in conjunction with specific primers corresponding to their respective non-coding sequences. A marked effect of chronic hypoxia on the expression of AT_1a and AT_1b subtypes was observed in the carotid body, whereas the expression levels of β-actin remained unchanged in chronic hypoxia when compared with that in controls (Figs 1 and 2). There were significant increases in the mRNA expression for the AT_1a subtype (Fig. 1A) and for the AT_1b subtype (Fig. 2A) in the chronic hypoxia group compared with their respective controls. The relative expression changes of AT_1a subtype (Fig. 1B) and AT_1b subtype (Fig. 2B) were about twofold in both subtypes, as demonstrated by RT-PCR and image analysis.

Effect of chronic hypoxia on the expression of AT_2 receptor subtype

The mRNA expression of AT_2 receptor was also studied in the carotid body. Chronic hypoxia had a less prominent effect on the AT_2 receptor expression (Fig. 3A). There was a slight but consistent increase of about 15% of the relative expression of AT_2 receptor in the chronically hypoxic group (Fig. 3B).

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Cellular localization of the AT$_1$ receptor by chronic hypoxia

To further elucidate the precise localization of AT$_1$ receptor expression and its upregulation during chronic hypoxia, immunohistochemistry was employed. Results from immunohistochemistry showed that there was an increase in the intensity of the immunoreactivity for the AT$_1$ receptor in chronically hypoxic carotid bodies compared with control (Fig. 4). Intense immunoreactivity for tyrosine hydroxylase was observed in the glomus cells, which was employed as a positive immunohistochemical marker for parenchymal type I glomus cells (Kameda et al. 1990). Tyrosine hydroxylase immunoreactivity was more intense in the hypoxic group (Fig. 4B) than in normoxic controls (Fig. 4A), as described previously (Wang et al. 1998). Detailed examination of consecutive sections of carotid body showed that distinct but less intense immunoreactivity for AT$_1$ receptor was predominantly localized to glomus cells in normoxic control (Fig. 4C,D) when compared with that in chronically hypoxic carotid body, which exhibited very intense immunostaining of AT$_1$ receptor (Fig. 4E and F). Intense immunostaining of AT$_1$ receptor was consistently observed in glomus cells of chronically hypoxic carotid body with sections counterstained with haematoxylin (Fig. 4G). Specificity of the immunostaining was validated by the negative control experiments when specific antibody was pre-absorbed in excess with its AT$_1$ receptor antigen either with counterstaining (Fig. 4H) or without counterstaining (Fig. 4I).

Increase of afferent nerve activities of the carotid body in vitro by Ang II

Ang II increased the resting activity of carotid bodies in normoxic and chronically hypoxic (CH) rats. Figure 5 shows that Ang II stimulated the afferent discharge of

Figure 2 (A) RT-PCR analysis of the mRNA expression of AT$_{1b}$ receptor in the rat carotid body. 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; Lane 5, AT$_{1b}$ expression in adrenal glands. 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. The data are expressed as means ± S.E.M. (n=5 for each group). *P<0.05.

Figure 3 (A) RT-PCR analysis of the mRNA expression of AT$_2$ receptor in the rat carotid body. Lane M, DNA marker; Lane 1, AT$_2$ receptor expression in control carotid bodies; Lane 2, AT$_2$ receptor expression in hypoxic carotid bodies; Lane 3, β-actin expression in control carotid bodies; Lane 4, β-actin expression in hypoxic carotid bodies; Lane 5, AT$_2$ expression in adrenal glands. The arrows indicate the expected size of amplified products from AT$_2$ receptor (511 bp) and β-actin (240 bp). (B) The relative expression of AT$_2$ receptor/β-actin mRNA. The data are expressed as means ± S.E.M. (n=5 for each group). *P<0.05.
Figure 4  Immunohistochemical localization of AT\textsubscript{1} receptor and tyrosine hydroxylase proteins in normoxic and chronically hypoxic carotid body of the rats. Distinct immunoreactivity for tyrosine hydroxylase was localized to the perinuclear cytoplasm of glomus cells in the carotid body from normoxia (A) and chronic hypoxia (B). Less intense immunoreactivity for the AT\textsubscript{1} receptor was predominantly localized to glomus cells from normoxic controls in lower magnification (C) and in high magnification (D). Intense immunostaining was consistently localized to glomus cells from chronic hypoxia in low magnification (E) and in high magnification (F). Distinct immunostaining in glomus cells from chronically hypoxic carotid body was shown in serial sections counterstained with haematoxylin (G). No immunostaining was observed in pre-adsorption of primary antibody with excess of AT\textsubscript{1} receptor antigen either with (H) or without (I) counterstaining. The bars represent 100 μm.
carotid body isolated from a CH rat. The perfusion of Ang II increased the basal discharge rate of a single unit (Fig. 5A–D). Ang II (0·1–100 nM) dose-dependently increased afferent discharge of the carotid body. The average dosage response to Ang II (100 nM) is 430% in the CH group compared with 220% in the normoxic group (Fig. 5E). Thus, the response to Ang II was enhanced in the CH group and the dosage–response curve for the CH group was shifted to the left, suggesting that maximal binding capacity for the Ang II was increased in the CH carotid body.

**Discussion**

The present study has clearly demonstrated the upregulation of the expression and function of Ang II receptor by chronic hypoxia in rat carotid body. Chronic hypoxia caused a marked increase in the expression of both AT1 and AT2 receptors, notably AT1 receptor as evidenced by RT-PCR analysis. Results from RT-PCR showed that chronic hypoxia significantly upregulates both mRNA expression for AT1a and AT1b subtypes. In parallel with the transcriptional level, the AT1 receptor protein was enhanced in expression, and the expression was specifically localized to the glomus cells of the carotid body, as demonstrated by immunohistochemistry. In addition, in vitro electrophysiological study coupled with specific antagonist for AT1 receptor further demonstrated that the upregulation of AT1 receptor expression was associated with the increased excitatory response to Ang II during chronic hypoxia. Results strongly suggest the increase in functional AT1 receptors in the carotid body during chronic hypoxia.

The present study is the first to present data on the upregulation of the expression and function of AT1 receptors by chronic hypoxia in carotid body by means of molecular biological, immunohistochemical and in vitro electrophysiological studies. Chronic hypoxia regulates the expression of RAS in a number of tissues, including the kidney (Neylon et al. 1996), the lung (Zhao et al. 1996),
the heart (Morrell et al. 1997) and the pancreas (Chan et al. 2000). In the carotid body, Ang II-binding sites have been demonstrated by in vitro autoradiography (Allen 1998). The present study provides evidence for the gene expression of both AT1 and AT2 receptors in the carotid body during normoxic condition. More importantly, our data showed that chronic hypoxia upregulates the expression of the AT1 receptors at both the gene and protein levels. The upregulation of the expression of Ang II receptors during chronic hypoxia appears to be subtype- and tissue-specific. Hence the upregulation in the carotid body was prominent in the AT1 but not AT2 subtype, whereas the major upregulation in the pancreas is the AT2 subtype (Chan et al. 2000). Moreover, it is more intriguing to demonstrate that chronic hypoxia can activate the expression of both AT1a and AT1b subtypes. Yet the relative importance of the subtypes for the AT1 receptor is currently unknown (Matsumbara et al. 1994) and the functional properties of the two subtypes are different in the signalling mechanisms (Tian et al. 1996). Their functional roles and upregulation in the carotid body remain to be elucidated.

The present study confirmed that the localization of the expression of AT1 receptors in the carotid body is mainly in the glomus cells. In addition, it is known that AT1 receptor may also express in the pre-synaptic terminals of sympathetic innervation (Castren et al. 1987). Consistent with previous finding that sympathetic denervation does not change the Ang II binding in the carotid body (Allen 1998), our results also suggests the sympathetic AT1 receptors if any would be in small amount in the carotid body. Glomus cells are believed to be the chemoreceptors and are the catecholamine-containing cells for the secretion of catecholamines during hypoxia (Fidone et al. 1982). In chronic hypoxia, the enzyme activity and the gene expression of the tyrosine hydroxylase increase, which in turn leads to increased production of catecholamine in the carotid body (Donnelly 1993). Indeed, our results also showed the enhanced tyrosine hydroxylase-immunoreactivity in the glomus cells with chronic hypoxia. Consistently, the upregulation of gene expression of the AT1 receptor was supported by the present observation that sympathetic denervation does not change the Ang II binding in the carotid body (Allen 1998). Following chronic hypoxia, the response to Ang II was elevated in the carotid body and the dose–response curve shifted leftward. This suggests that the affinity to Ang II binding or the receptor density is enhanced in the chronic hypoxic group. The latter possibility is supported by the fact that the gene expression of the AT1 receptor in the carotid body was increased in chronic hypoxia. A previous report has shown that the AT1 receptor is the major subtype, which mediates the carotid body response to Ang II in normoxic rats (Allen 1998). In chronic hypoxia, the response to Ang II is inhibited by losartan, a specific antagonist for AT1 receptor. This suggests that the AT1 receptor mediates the response to Ang II and that the receptor is upregulated in the carotid body during chronic hypoxia. Hence, the upregulation of AT1 receptors in the carotid body during chronic hypoxia is supported by the present observation that the intensity of AT1-immunoreactivity was higher in the chronically hypoxic group than in the normoxic control. This finding suggests that chemoreceptors increase the density of functional AT1 receptors in chronic hypoxia.

In vitro electrophysiological studies further elucidated that Ang II increased afferent discharge of the carotid body. This finding is consistent with a previous report that Ang II stimulated carotid chemoreceptor activity (Allen 1998). Following chronic hypoxia, the response to Ang II was elevated in the carotid body and the dose–response curve shifted leftward. This suggests that the affinity to Ang II binding or the receptor density is enhanced in the chronic hypoxic group. The latter possibility is supported by the fact that the gene expression of the AT1 receptor in the carotid body was increased in chronic hypoxia. A previous report has shown that the AT1 receptor is the major subtype, which mediates the carotid body response to Ang II in normoxic rats (Allen 1998). In chronic hypoxia, the response to Ang II is inhibited by losartan, a specific antagonist for AT1 receptor. This suggests that the AT1 receptor mediates the response to Ang II and that the receptor is upregulated in the carotid body during chronic hypoxia. Hence, the upregulation of AT1 receptors during chronic hypoxia is supported by the present observation that the intensity of AT1-immunoreactivity was higher in the chronically hypoxic group than in the normoxic control. This finding suggests that chemoreceptors increase the density of functional AT1 receptors in chronic hypoxia.
elevates the sensitivity and physiological response of the carotid body to Ang II stimulation. However, the mechanism underlying the upregulation of the AT$_1$ receptor in chronic hypoxia remains undefined and merits further investigation.

In summary, the present study has provided evidence that chronic hypoxia could upregulate the expression of Ang II receptors, predominantly the AT$_1$ receptor, which might result in enhancing the carotid afferent nerve activity to Ang II stimulation. This could, in turn, increase the cardiorespiratory response and regulate the salt and water homeostasis during the stress of chronic hypoxia.

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