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ORIGINAL ARTICLE

Man-Lung Fung · Siu-Yin Lam · Yueping Chen
Xiaoli Dong · Po Sing Leung

Functional expression of angiotensin II receptors in type-I cells of the rat carotid body

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Abstract Angiotensin II increases afferent discharge from the carotid body in vitro. We hypothesized that angiotensin II receptors (AT receptors) are expressed functionally in the type-I cell of the carotid body. Cytosolic free [Ca^{2+}] ([Ca^{2+}]$_i$) in type-I cells freshly dissociated from rat carotid bodies was measured spectrofluorimetrically. Angiotensin II (10–100 nM) concentration-dependently increased [Ca^{2+}]$_i$ in type-I cells. The [Ca^{2+}]$_i$ response was blocked by pretreatment with losartan (1 μM), an AT_1 receptor antagonist, but not by blockade of AT_2 receptors with PD-123319 (1 μM). Moreover, the gene expression of AT_1 receptors was assessed by the reverse transcriptase polymerase chain reaction and gene transcripts of both AT_{1a} and AT_{1b} receptors were detected in the carotid body. In addition, immunohistochemical study revealed that AT_1 immunoreactivity was localized in lobules of type-I cells in the carotid body. Taken together, these results suggest that type-I cells in the rat carotid body express functional angiotensin II receptors. The binding of angiotensin II to the AT_1 receptors increases [Ca^{2+}]$_i$, a key step of the intracellular signalling cascade following the activation of the receptors. It is concluded that angiotensin II modulates carotid body chemoreceptor function directly via AT_1 receptors in the type-I cell.

Keywords Angiotensin II · Angiotensin II receptor · AT_{1a} · AT_{1b} · Carotid body · Glomus cell · Type-I cell · Respiration

M-L. Fung (✉) · Y. Chen · X. Dong
Department of Physiology and Institute
of Cardiovascular Science and Medicine,
Faculty of Medicine, University of Hong Kong,
5 Sassoon Road, Pokfulam, Hong Kong SAR
e-mail: fungml@hkucc.hku.hk
Tel.: +852-28199234, Fax: +852-28559730

S-Y. Lam · P.S. Leung
Department of Physiology, Chinese University of Hong Kong,
Shatin, Hong Kong SAR

Introduction

The carotid body plays a pivotal role in the transduction of chemical signals in arterial blood to sensory afferent discharges projecting to brainstem nuclei for the modulation of cardiorespiratory performance. Type-I cells in the carotid body are believed to be the receptor cells for peripheral chemoreception and their activity is associated closely with the cytosolic free [Ca2+] ([Ca^{2+}]$_i$). The elevation of [Ca^{2+}]$_i$ may be coupled to the vesicular secretion of catecholamines and is believed to be an important step for chemotransduction [13]. Hence, intracellular calcium changes with acute hypoxia and histotoxic hypoxia in type-I cells dissociated from rabbit and rat carotid bodies [5, 6, 8, 9, 12, 31].

Angiotensin II (ANG II) is an octapeptide that acts on a variety of tissues and cell types. It is a potent arteriolar constrictor and is the major circulating hormone for stimulating aldosterone excretion from the adrenal cortex [26]. Interestingly, peripheral infusion of ANG II stimulates respiration [24, 27]. The site of this action of ANG II is not clear, but may be the circumventricular organs of the brain because ANG II does not pass the blood brain barrier [25, 30].

ANG II receptors (AT receptors) are found in a wide spectrum of organs and tissues [20]. Two subtypes, AT_1 and AT_2, have been identified by their distinct affinities for specific pharmacological blockers [29]. AT_1 and AT_2 receptors have been cloned and are members of the seven-transmembrane-spanning, guanine nucleotide-binding protein (G protein)-coupled receptor family [21, 22, 28]. An intracellular signalling pathway following the activation of ANG II receptors has been proposed for some cell types [7]. In adrenal glomerulosa cells, ANG II binding to the AT_1 receptor stimulates G protein activity and the phosphatidylinositol/phospholipase C (PLC) pathway in the plasma membrane and leads to the formation of 1,2-diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP_3), which releases the intracellular calcium store and elevates [Ca^{2+}]$_i$ [3, 4].

Recently, Allen [1] has reported that ANG II modulates the afferent activity of the carotid body and that this

modulation is independent of the haemodynamic effect of ANG II, since ANG II increases the resting afferent discharge in the isolated carotid body in vitro. The ANG II action is presumably via AT_1 because an AT_1 antagonist attenuates the effect of ANG II on the carotid body [1]. In addition, an autoradiographic study has shown ANG II binding sites to be present in the carotid body [1].

Given that ANG II modulates the afferent activity of the carotid body, we hypothesized that the neural response to ANG II is mediated by the AT_1 receptor expressed in the type-I cell of the carotid body. We propose that ANG II increases $[Ca^{2+}]_i$ in the type-I cells via the activation of the AT_1 receptor. The aims of the study were thus to determine the $[Ca^{2+}]_i$ response to ANG II in type-I cells dissociated from the rat carotid body, to examine the transcriptional expression of AT_1 receptors in the carotid body and to determine the localization of AT_1 receptors in the carotid body.

Materials and methods

Isolation of the carotid body

The experimental protocol for this study was approved by the Committee on the Use of Live Animals in Teaching and Research of The University of Hong Kong. Following deep anaesthesia with halothane, Sprague-Dawley rats (70–80 g) were decapitated and the carotid body dissected free from the bifurcation and the superior sympathetic ganglion in chilled rat Ringer oxygenated with 95% O_2 and 5% CO_2. The carotid body was then incubated in collagenase and protease for 30 min at 35±1 °C [16].

Dissociation of carotid body type-I cells

Following enzymatic treatment, the carotid body cells were dispersed by triturating with glass pipettes [12]. Fura-2 acetoxymethyl ester (fura-2AM, Molecular Probes, Eugene, Ore., USA; 5 µl, final concentration 5 µM) was added and gently mixed with the cells in 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES)-Ringer (1 ml) and the cells incubated for 30 min in the dark at room temperature. The cells were then centrifuged at 200 *g* for 5 min and prepared for the spectrofluorimetric measurement of $[Ca^{2+}]_i$ [6, 31]. Type-I cells in clusters of 8–20 cells were studied and the morphological criteria for their confirmation as type-I cells was according to the methods used in a previous study [12].

Spectrofluorimetric measurement

$[Ca^{2+}]_i$ was measured in fura-2-loaded type-I cells freshly dissociated from rat carotid bodies. The cells were seeded on a cover-slip placed on the stage of an inverted microscope equipped with a dual-wavelength excitation spectrofluorimeter. The cells were perfused with HEPES buffer at 0.5 ml/min at room temperature (~22 °C). Background fluorescence intensity was subtracted from the signals. Fluorescent signals were obtained at 340 and 380 nm excitation wavelengths. The ratio of the fluorescence intensity (340/380) was used to estimate $[Ca^{2+}]_i$ in the type-I cells. The $[Ca^{2+}]_i$ 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} the fluorescence ratio at zero Ca^{2+}, R_{max} the fluorescence ratio at saturated Ca^{2+}, K_d the dissociation constant for fura-2 (224 nM) and β the ratio of fluorescence intensity (380 nm) at zero Ca^{2+} to fluorescence intensity (380 nm) at saturated Ca^{2+}.

Experimental paradigm

Concentration dependence was determined by the $[Ca^{2+}]_i$ response to ANG II at 10, 50 and 100 nM. ANG II was injected as a bolus into the 0.5-ml bath. The antagonist for AT_1, losartan (1 µM), or for AT_2, PD123319 (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.

Reverse transcriptase polymerase chain reaction (RT-PCR)

Carotid bodies were dissected free and frozen at –70 °C. About 40–50 carotid bodies were collected for each RT-PCR experiment. Total ribonucleic acid (RNA) was isolated from the tissue using the acid guanidinium thiocyanate-phenol-chloroform protocol [10]. Briefly, tissues were homogenized in 4 M guanidinium thiocyanate solution and extracted repeatedly with water-saturated phenol. After extraction with chloroform, RNA was precipitated by isopropanol. The resultant pellet was finally resuspended in water with diethylpyrocarbonate. The total RNA was studied by gel electrophoresis and quantified by spectrophotometry. Total RNA (10 µg) was subjected to first-strand complementary deoxyribonucleic acid (cDNA) synthesis using random hexamer primers and Superscript II transcriptase (Gibco-BRL, 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 used directly for PCR amplification. Messenger RNAs (mRNAs) of the AT_1 subtypes, i.e. AT_{1a} and AT_{1b}, were detected with primers as employed previously [17]. β-Actin mRNA was detected with specific primers corresponding to the rat cytoplasmic nucleotide sequence [23]. All RNA was shown to be free of DNA contamination by RT-PCR without addition of reverse transcriptase. All samples were analysed for both ANG II receptor subtypes and β-actin genes in the logarithmic phase of the amplification reactions. PCR reactions were carried out in a volume of 50 µl containing the corresponding sense and antisense primer sequences using the PCR Reagent System (Gibco-BRL). The PCR conditions were 30 cycles of denaturing at 94 °C for 1 min; annealing at 58 °C (AT_{1a}) or 62 °C (AT_{1b}) for 1 min and elongating at 72 °C for 2 min. The amplified mixture (10 µl) was separated on 2% agarose gel electrophoresis and the amplified DNA bands detected by ethidium bromide staining.

Immunohistochemistry

Carotid bifurcations were excised from rats under deep anaesthesia. The bifurcation was rinsed in phosphate buffered saline and frozen in isopentane. Consecutive cryosections (8–10 µm) were cut on a cryotome and mounted on gelatine-coated slides. Sections were fixed in 4% paraformaldehye for 30 min and processed for immunoperoxidase staining as described previously [18]. Briefly, sections were rinsed in methanol containing 0.3% H_2O_2 for 30 min and incubated overnight at 4 °C with anti-AT_1 receptor serum (Santa Cruz Biotech, Santa Cruz, Calif., USA), diluted to 0.2 µg/ml. The primary antibody was detected using an avidin-biotin conjugate kit (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, Calif., USA) and the sections reacted in Vector VIP substrate kit (Vector Laboratories) for 4–8 min to visualize the AT_1 immunoreactivity, which stained purple. Some sections were slightly counterstained with haematoxylin and then dehydrated and mounted. The specificity of the AT_1 immunoreactivity was determined by substitution of buffer for the primary antibody and

liquid phase preadsorption of the primary antibody with excess blocking peptide (Santa Cruz Biotech).

Materials and pharmacological agents

The rat Ringer solution contained (mM): NaCl 125, KCl 3.1, $NaHCO_3$ 26, NaH_2PO_4 1.25, $MgSO_4$ 1.3, $CaCl_2$ 2.4, D-dextrose 10. The HEPES-Ringer contained (mM): NaCl 140, KCl 3, NaH_2PO_4 1.25, $MgCl_2$ 1, $CaCl_2$ 1, HEPES 10, D-dextrose 25 (pH=7.35–7.4). Antagonists for ANG II receptors were dissolved in HEPES-Ringer solution. The concentrations of ANG II and sodium cyanide were 5 µM and 1 M, respectively, for the injection. Fura-2AM was dissolved in dimethylsulphoxide (DMSO) in 1 mM stock. Drugs were purchased from Sigma (St. Louis, Mo., USA) or as otherwise noted.

Data Analysis

For the $[Ca^{2+}]_i$, the resting and peak values of the fluorescence ratio of (340/380 nm) or calibrated $[Ca^{2+}]_i$ (in nanomole/litre) of the responses during drug treatment were calculated. Values were normalized as a percentage of control if necessary and presented as mean±SE. Means were compared with the paired *t*-test or the non-parametric Wilcoxon signed-rank test (comparison of pre- and post-treatment). ANOVA with a post-hoc test (Dunnett's *t*-test) was used for multiple comparisons of values in drugs studies among groups with different concentrations. Differences were considered significant at $P<0.05$.

Results

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

Of 85 clusters of type-I cells, 33 (38.8%) responded to ANG II (10–100 nM). ANG II increased $[Ca^{2+}]_i$ in all of the responsive clusters of cells (Fig. 1A and B). The peak response was reached shortly following ANG II stimulation and $[Ca^{2+}]_i$ gradually returned to the resting level within 3 min (Fig. 1A and B). On average, the increase in $[Ca^{2+}]_i$ in response to ANG II (100 nM) was 30.5±4.9 nM (n=24). In addition, different concentrations of ANG II (10, 50, 100 nM) were tested in 12 clusters of type-I cells (Fig. 1B). The $[Ca^{2+}]_i$ increase in response to ANG II was concentration dependent (Fig. 1C).

Blockade of ANG II receptor subtypes

Losartan (1 µM) treatment per se did not change the resting $[Ca^{2+}]_i$ (P=0.1, paired *t*-test, n=6) but it largely abolished the $[Ca^{2+}]_i$ response to ANG II in the type-I cells (Fig. 2A). The ANG II-induced $[Ca^{2+}]_i$ peak changes in pre- and post-treatment were significantly different (P=0.03, paired *t*-test, n=6) (Fig. 2C). In contrast, PD123319 did not cause any significant difference in the peak $[Ca^{2+}]_i$ response to ANG II (P=0.2, n=6) (Fig. 2C), nor did the treatment per se change the $[Ca^{2+}]_i$ (P=0.5, paired *t*-test, n=6). Moreover, vehicle treatment did not change the $[Ca^{2+}]_i$ response to ANG II stimulation (P=0.1, Wilcoxon signed-rank test, n=3).

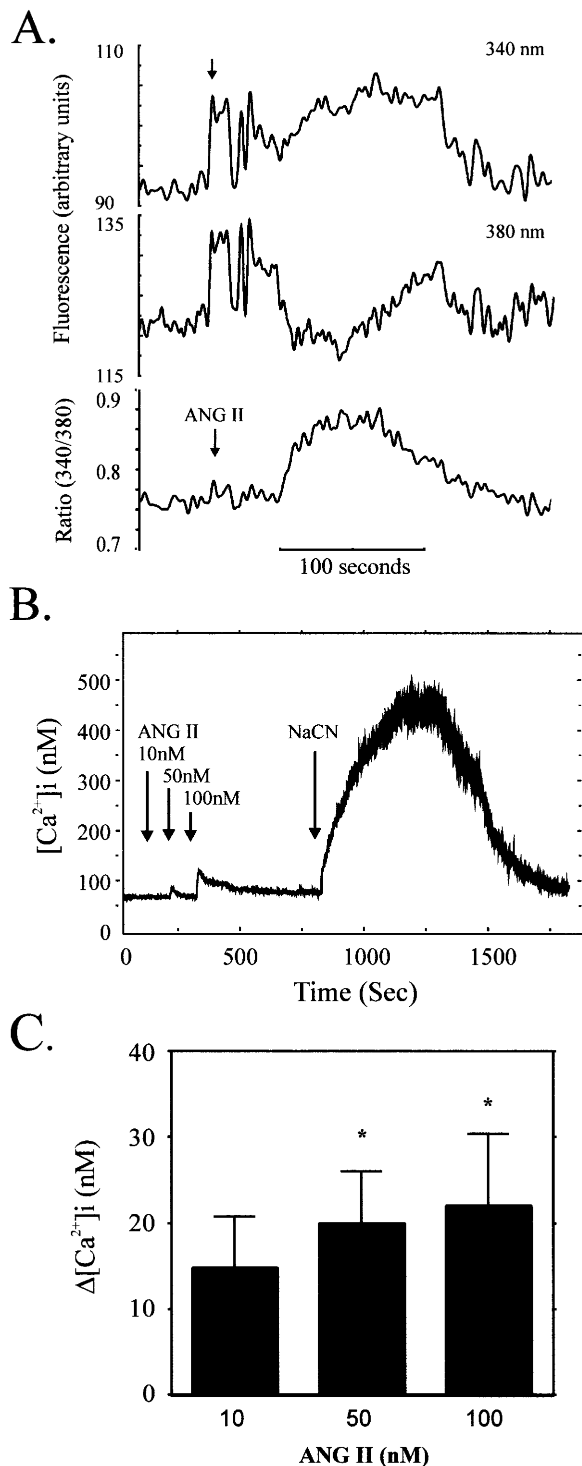

Fig. 1 **A** Fura-2 fluorescence signal at 340 (*upper trace*) and 380 (*middle trace*) nm and the ratio of these (*lower trace*) in clusters of between eight and ten dissociated type-I cells of the rat carotid body, showing the response to the addition of 100 nM angiotensin II (*ANG II*) to the bath. An increase in the ratio indicates an increase in cytosolic free $[Ca^{2+}]$ ($[Ca^{2+}]_i$). Artefacts of drug application are seen as in the sharp increases of fluorescence intensity at both wavelengths (*arrow*). **B** ANG II (10–100 nM) and histotoxic hypoxia with cyanide (NaCN, 2 mM) both increased the $[Ca^{2+}]_i$ of the dissociated type I cells. **C** Concentration/response relationship for ANG II-induced $[Ca^{2+}]_i$ increases ($\Delta[Ca^{2+}]_i$) in type-I carotid body cells (n=12). *$P<0.05$ vs. 10 nM ANG II

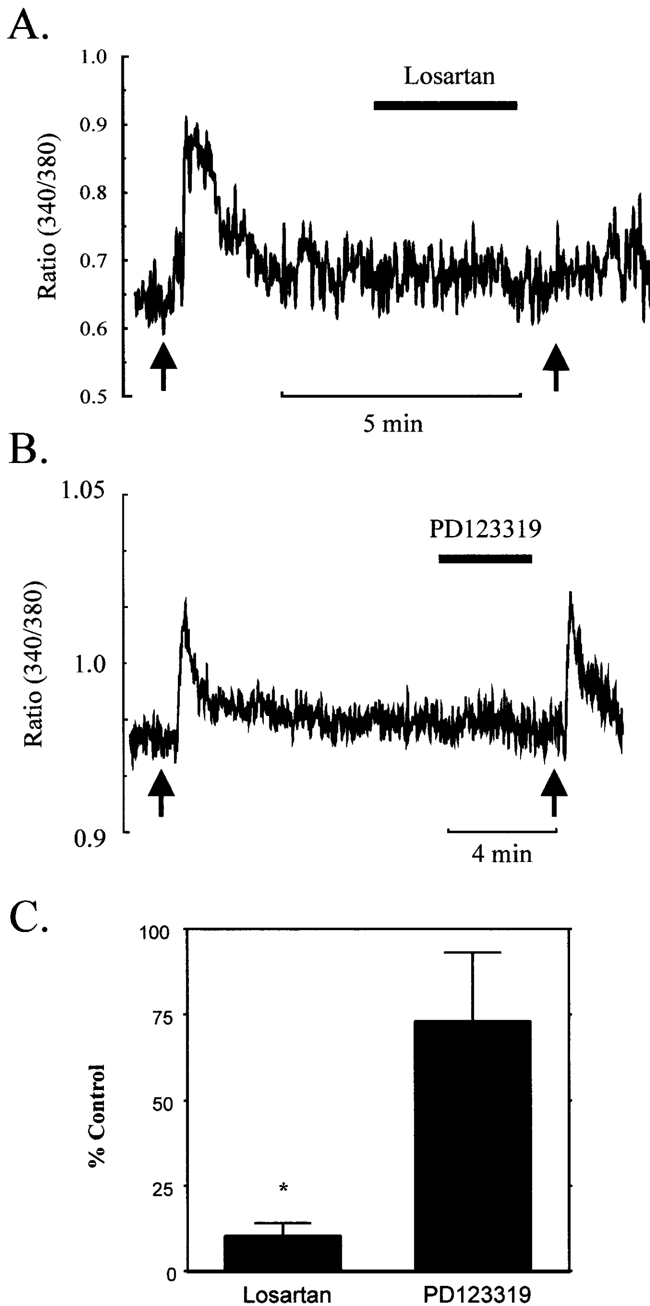

Fig. 2A–C Blockade of AT_1 but not AT_2 receptor subtypes attenuates the ANG II-induced $[Ca^{2+}]_i$ change. **A** Effect of the AT_1 antagonist losartan (perfusion for 3 min, *bar*) on $[Ca^{2+}]_i$ response to ANG II (100 nM, *arrows*). **B** Lack of effect of the AT_2 antagonist PD123319 (perfusion for 3 min, *bar*) on the $[Ca^{2+}]_i$ response to ANG II (100 nM, *arrows*) in a cluster of type-I cells. **C** Summary of effects of losartan and PD123319 on the ANG II-induced $[Ca^{2+}]_i$ change. The $[Ca^{2+}]_i$ response to ANG II following the drug treatment is normalized to that of the pretreatment (*% Control*); mean±SE. $^*P<0.05$ vs. corresponding pretreatment group

Transcriptional expression of the AT_1 receptors in the carotid body

To determine the mRNA expression of AT_1 receptors in the rat carotid body, the gene transcripts of AT_1 subtypes

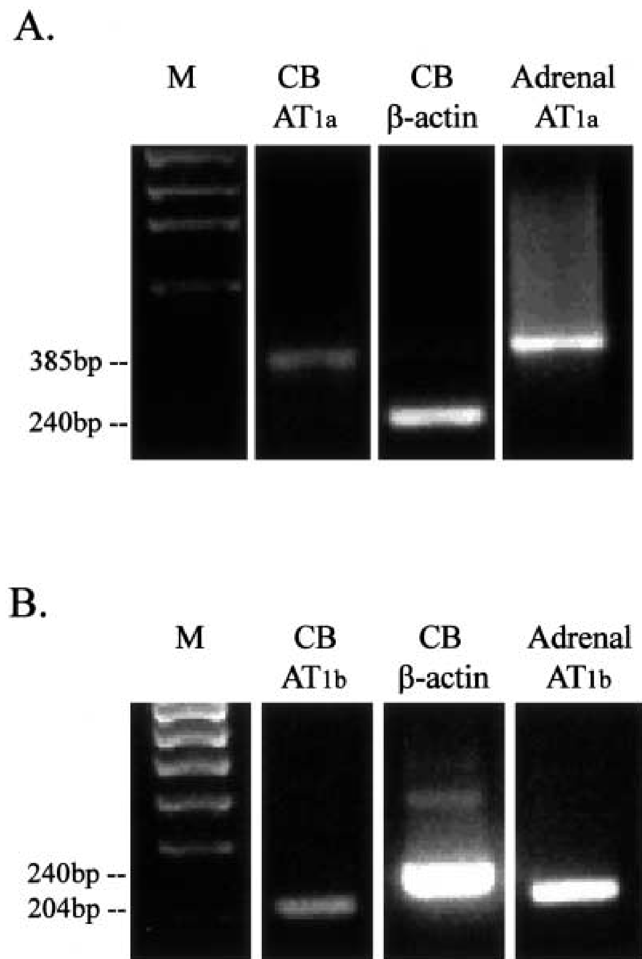

Fig. 3A, B Messenger ribonucleic acid (mRNA) expression of AT_1 receptors in the carotid body. **A** The polymerase chain reaction (PCR) product of AT_{1a} (385 bp) in rat carotid bodies (*CB*) is shown in *lane 2*. The AT_{1a} expressed in the adrenal gland is shown as a positive control (*adrenal*, *lane 4*). The internal control for the reverse transcription (RT)-PCR study is β-actin (240 bp) in the carotid bodies (*lane 3*). A deoxyribonucleic acid (DNA) marker (*M*) is in *lane 1*. **B** The PCR product of AT_{1b} (204 bp) in the rat CB is shown in *lane 2*. The AT_{1b} expressed in the adrenal gland is shown as a positive control (*adrenal*, *lane 4*). The internal control for the RT-PCR study is β-actin (240 bp, *lane 3*). A DNA marker (*M*) is in *lane 1*

AT_{1a} and AT_{1b} were detected by RT-PCR using specific oligonucleotide primers based on the corresponding genes as reported previously [17]. The RT-PCR products of AT_{1a} (385 bp) and AT_{1b} (204 bp) were found in carotid bodies (Fig. 3), indicating the mRNA expression of AT_1 receptors. As a positive control, the expression of AT_{1a} and AT_{1b} was shown in the adrenal gland. The internal control for the RT-PCR study was the β-actin expression (240 bp) in the carotid bodies.

Localization of AT_1 receptors in the carotid body

To determine the localization of AT_1 receptors in the rat carotid body, AT_1 immunoreactivity was detected by us-

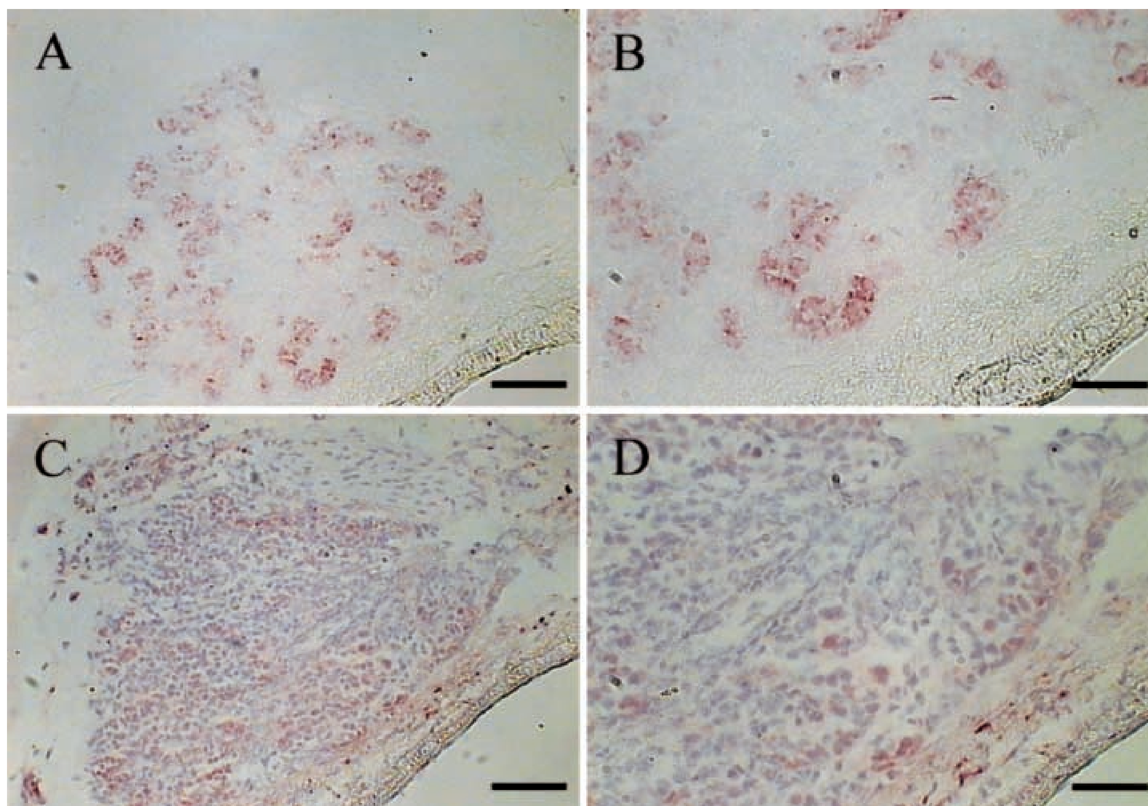

Fig. 4A–D AT_1-immunoreactivity in the rat carotid body. **A** Low magnification (20×) showing the faint-to-moderate immunostaining in the carotid body parenchyma. **B** High magnification (40×) showing localization of staining scattered in lobules of the carotid body. **C**, **D** Consecutive sections of the carotid body treated by preabsorption of the primary antibody and counterstained with haematoxylin at low (**C**) and high (**D**) magnification. Calibration *bars* are 40 μm at low and 80 μm at high magnification

ing a receptor-specific antibody. Faint-to-moderate positive immunostaining was observed in the carotid body parenchyma (Fig. 4). The staining was scattered in lobules of type-I cells (Fig. 4). Neither preabsorption of the antibody nor replacement of the antibody with buffer yielded positive immunostaining in the carotid body sections (Fig. 4).

Discussion

This is the first study to demonstrate that ANG II increases $[Ca^{2+}]_i$ in type-I cells of the rat carotid body, that gene transcripts of AT_{1a} and AT_{1b} receptors are expressed in the rat carotid body and that AT_1 receptors are localized in the lobules of type-I cells. Taken together, these results support the hypothesis that ANG II receptors are expressed functionally in the type-I cell of the carotid body. In addition, the $[Ca^{2+}]_i$ response to ANG II was blocked by an AT_1 but not by an AT_2 antagonist, suggesting that ANG II binding to AT_1 receptors stimulates the intracellular signalling pathway and elevates the $[Ca^{2+}]_i$ in the type-I cells. In turn, the $[Ca^{2+}]_i$ elevation may increase afferent discharge of the carotid body and contribute to the ventilatory and cardiovascular responses to physiological stimulation.

The $[Ca^{2+}]_i$ response to ANG II via AT_1 receptors in type-I cells of the rat carotid body

ANG II induced a transient elevation of $[Ca^{2+}]_i$ in type-I cells. The $[Ca^{2+}]_i$ response to ANG II was seen only in a fraction (38%) of the clusters of cells. This suggests that a subpopulation of type-I cells expresses the AT_1 receptor. This may explain the high variability in the magnitude of the multi-unit responses to ANG II reported in a previous electrophysiological study [1]. Heterogeneity of type-I cells has also been described in their responses to acetylcholine. Dasso et al. have reported that acetylcholine increases $[Ca^{2+}]_i$ in 55% of type-I cells and that the responses to nicotinic and muscarinic stimulation are different [11]. The functional significance of the phenotypic heterogeneity of the type-I cells remains unknown.

The $[Ca^{2+}]_i$ response to ANG II was relatively small compared with the response to cyanide (Fig. 1). This observation is consistent with the electrophysiological finding that the increased afferent discharge induced by ANG II is less than that induced by hypoxia [1]. We believe that the $[Ca^{2+}]_i$ response is specific to the activation of ANG II receptors because the $[Ca^{2+}]_i$ response to ANG II was abolished by losartan pretreatment which antagonizes ANG II binding to the AT_1 receptors. In contrast, the $[Ca^{2+}]_i$ response was only slightly decreased by an AT_2 receptor antagonist and was not changed by

vehicle treatment. These results strongly suggest that the activation of AT_1 receptors mediates $[Ca^{2+}]_i$ elevation in type-I cells.

It is generally accepted that $[Ca^{2+}]_i$ elevation is critical for type-I cell secretion and chemotransduction [6, 9]. Indeed, we found the $[Ca^{2+}]_i$ elevation in all of the cells responsive to ANG II, suggesting the $[Ca^{2+}]_i$ elevation is involved in the increase of chemoreceptor afferent activity. The AT_1 receptor is a G protein-coupled receptor and its activation may lead to the release of intracellular Ca^{2+} via the IP_3 pathway [3, 4]. The details of the signalling pathway for the activation of AT_1 receptors in the type-I cells remain to be elucidated.

Transcriptional and posttranscriptional expression of AT_1 receptor subtypes in rat carotid body

Although ANG II receptors are expressed in various cell types, mRNA expression of the ANG II receptor in the carotid body was undefined. In addition, two subtypes of the AT_1 receptor are known to be expressed in the rat in a tissue-specific manner [15]. We found both AT_{1a} and AT_{1b} receptors in the rat carotid body. This finding supports the previous autoradiographic findings that a large number of AT_1 receptors are present in the carotid body [1]. AT_1 receptor subtypes may play different physiological roles in the carotid body although the functional difference, if any, between AT_{1a} and AT_{1b} receptors is not clear at the moment and pharmacological tools are not yet available to distinguish between these subtypes.

The localization of the AT_1 receptor expression has not been determined previously and any functional implications might require information on the fine detail of such localization. We found that AT_1-immunoreactivity to be localized in lobules of the carotid body, suggesting that AT_1 receptors are expressed in the type-I cell. This finding supports previous autoradiographic findings that AT_1 receptors are intrinsic to the type-I cells [1]. In addition, AT_1 immunoreactivity was scattered in the lobules of type-I cells. This is consistent with our findings in functional study that AT_1 receptors were expressed in a subpopulation of the type-I cells.

Functional implications of the AT_1 receptors in the carotid body

It is well known that the renin-angiotensin system plays an important role in the hormonal regulation of salt and water balance in the body [26]. Physiological stimuli, such as changes in extracellular fluid volume, osmolarity, blood volume or sodium depletion stimulate the renin-angiotensin system and thus increase the plasma ANG II level [19]. Our results suggest that the carotid body directly responds to circulating ANG II and thus induces carotid chemoreceptor activity that activates the neural regulation of cardiovascular and respiratory functions. In addition, increased carotid chemoreceptor activity elevates renal sympathetic activity and this increases renin secretion by the juxtaglomerular cells in the kidney, which would further activate the renin-angiotensin system to increase sodium reabsorption and water intake [14]. Hence, our results suggest that, in addition to ANG II-sensitive neurons in the circumventricular organs of the brain [2], circulating ANG II can also excite peripheral chemoreceptors that in turn elicit cardiorespiratory changes and facilitate salt and water balance.

In conclusion, this study has demonstrated the functional expression of AT_1 receptors in the type-I cells of the rat carotid body. ANG II binding to the AT_1 receptors elevated $[Ca^{2+}]_i$ activity of the type-I cells and the increased $[Ca^{2+}]_i$ activity may modulate carotid body function.

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