Regulation of the angiotensin-converting enzyme activity by a time-course hypoxia in the carotid body

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Lam, Siu Yin, Man-Lung Fung, and Po Sing Leung. Regulation of the angiotensin-converting enzyme activity by a time-course hypoxia in the carotid body. J Appl Physiol 96: 809–813, 2004. First published October 3, 2003; 10.1152/japplphysiol.00684.2003.—Chronic hypoxia activates a local angiotensin-generating system in the carotid body. Here, we test the hypothesis that the activity of the critical enzyme for this system, angiotensin-converting enzyme (ACE), in the carotid body is subject to regulation by a time-course hypoxia. Results from the carotid body assays showed that ACE activity was markedly increased under the hypoxic stress of 7-, 14-, 21-, and 28-day exposures. The changes in ACE activity of 7-day (15.00 vs. 30.95 \times 10^{-5} \text{ nmol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}), 14-day (8.73 vs. 30.25 \times 10^{-5} \text{ nmol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}), and 21-day (11.41 vs. 31.83 \times 10^{-5} \text{ nmol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}) hypoxia treatments were enhanced significantly. However, ACE activity in 28-day (13.18 vs. 24.53 \times 10^{-5} \text{ nmol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}) hypoxia treatment was observed to increase insignificantly when compared with results in the respective control groups. Captopril inhibited all rises in ACE activity in both the control and experimental groups. Results clearly indicate an activation of the enzymatic activity of ACE, the critical enzyme for determining the conversion of angiotensin I into the physiologically active angiotensin II, by chronic hypoxia in the carotid body. An increase in the ACE activity may increase the local production of angiotensin II in the carotid body and thus its agonist action at the AT1 receptor. This may be important in the modulation of cardiopulmonary adaptation in the hypoxic ventilatory response as well as for electrolyte and water homeostasis during chronic hypoxia.

HIGHLIGHTED TOPIC | Oxygen Sensing in Health and Disease

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present study was designed specifically to examine the hypothesis that the activity of this critical enzyme, which is responsible for determining the generation of physiologically active ang II, is regulated by a time-course quenched fluorogenic substrate, methoxycarbonyl (Meoc)-dl-Amp-Gly-Lys[dinitrophenylhydrazine (ε-DNP)]-Gln-OH.

**MATERIALS AND METHODS**

**Experimental animals and isolation of carotid body.** Adult male Sprague-Dawley rats aged 28 days were used. The animals were bred and raised under pathogen-free conditions, with a controlled ambient temperature of 20 ± 1°C, relative humidity of 60–80%, and a 14:10-h light-dark cycle in the Laboratory Animal Services Centre of the Chinese University of Hong Kong. Standard rat chow and tap water were supplied ad libitum. Ethical approval for the animal model and experimental protocols were obtained from the Animal Experimentation Ethics Committee of the Chinese University of Hong Kong, Hong Kong. After a deep anesthesia with halothane, Sprague-Dawley rats were decapitated, and carotid bodies were dissected from the bifurcation and the superior sympathetic ganglion in PBS, pH 7.4.

**Chronic hypoxia rats.** In the rat model of chronic hypoxia, the animals were kept in a moderate level (10%) of oxygen, which is relevant to clinical and physiological situations as described previously (17, 27, 32). Briefly, experimental rats were exposed to an isobaric hypoxic chamber for a period of up to 4 wk. The oxygen level was continuously monitored by an oxygen analyzer (Vacumetrics) and was maintained by a servo-feedback control of solenoid valves that gated the inflow of pure nitrogen. The nitrogen was then allowed to mix with room air that was generated into the chamber by a pump at a speed of 1–2 l/min. Inside of each chamber, desiccator and soda lime were used to maintain the humidity and carbon dioxide condition. Each chamber was opened for ~15 min for regular maintenance every 2–3 days. To examine the time-course effect of hypoxic treatment on experimental rats, animals aged 28 days were conditioned in the above environment for 7, 14, 21, and 28 days. Time- and age-matched rats kept in the same housing but with normal room air supply were used as the normoxic controls.

**Specific assay for ACE activity.** The purification and characterization of microvillar membrane-bound ACE from porcine kidney cortex were described previously (20). A specific assay for ACE activity that uses an internally quenched fluorogenic substrate, Meoc-dl-Amp-Gly-Lys[ε-DNP]-Gln-OH, has been recently developed (22). Briefly, an internally quenched tetrapeptide substrate for ACE fluorogenic that used the fluorescent amino acid 2-amino-3-(7-methoxy-4-coumaryl) propanoic acid, quenched by a dinitrophenyl group, was attached to a lysine residue. The peptide [N-Meoc-dl-Amp-Gly-Lys(ε-DNP)-Gln-OH] was synthesized and characterized as reported previously (22).

To prepare membrane protein for measurement of ACE activity, 10 carotid bodies obtained from five animals were pooled for an assay. The carotid bodies and lungs were homogenized in 1.9 (wt/vol) PBS (pH 7.4) containing 1 mM PMSF (28). The homogenate was subject to sedimentation at 1,000 g at 4°C for 20 min. The membrane fraction within the supernatant portion was collected by centrifugation at 30,000 g for 30 min at 4°C. The protein concentration was measured by the Bio-Rad protein assay. The measurement of ACE activity was performed as described previously (22). Briefly, membrane proteins (5 μg) were preincubated with Tris buffer (100 mM Tris-HCl, pH 8.0, and 300 mM NaCl) in a final volume of 950 μl at 37°C for 30 min. For the control setting, 15 mM captopril (Sigma Chemical, St. Louis, MO) was added to inhibit the ACE activity. After preincubitation, 50 μl of 0.4 mM fluorogenic ACE substrate (0.32 mg/ml in Tris buffer) were added to the mixtures and incubated again at 37°C for 60 min. The fluorescence of the mixtures was measured by a fluorometer. A mixture of 50 μl of ACE substrate and 950 μl of Tris buffer was used as the blank and was used to set zero with excitation at 328 nm and emission at 392 nm of the fluorometer. Fluorescent standard of 0.1 mM (37.8 mg/l in Tris buffer) was diluted to give 1 μM solution as a reference reading for calculating the ACE activity (in nmol/μg of protein min−1).

**Statistical analysis.** ACE activities were expressed as nanomoles per micrograms of protein per minute. Results were expressed as means ± SE (n = 5 assays) for the controls and the chronically hypoxic groups. Differences were compared by an unpaired t-test, analyzed by one-way ANOVA, and followed by Duncan’s multiple range test to detect intergroup differences. P values of <0.05 were considered significant.

**RESULTS**

The changes of enzymatic ACE activities in the carotid body by chronic hypoxia were investigated by using a specific assay for ACE activity. The activities of carotid body ACE in each group of experiments were calculated in (nmol/μg min−1 × 10−5), and lungs were collected from the normoxic rats and used as a positive control for the expression of ACE activity in the present study. The effects of chronic hypoxia on the ACE activities were observed in a time-dependent manner with 7-, 14-, 21- and 28-day hypoxia, when compared with their respective normoxic controls (Table 1). With the use of an internally quenched fluorogenic substrate, ACE activities in rat carotid bodies that underwent 7-, 14-, 21-, and 28-day treatment under normoxic condition were found to be 15.00 × 10−5, 8.73 × 10−5, 11.41 × 10−5, and 13.18 × 10−5 nmol/μg min−1, respectively. Chronic hypoxia markedly increased the ACE activity of rat carotid bodies up to 30.95 × 10−5, 30.25 × 10−5, 31.83 × 10−5, and 24.53 × 10−5 nmol/μg min−1 in the 7-, 14-, 21- and 28-day treatments, respectively. The specific activities of ACE in the 7-day (Fig. 1), the 14-day (Fig. 2), and the 21-day (Fig. 3) hypoxic rat carotid bodies were shown to be significantly different from their respective normoxic controls; however, the 28-day hypoxic carotid bodies showed an insignificance difference compared with results from their normoxic controls (Fig. 4). Almost no ACE activity was detected when captopril, a specific ACE inhibitor, was added to any of the samples tested, both in the carotid bodies and in the lung tissues (Figs. 1–4). This clearly shows that the intrinsic peptidase present in the carotid body is a specific substrate for ACE. There were ~2.06, 3.47, 2.79, and 1.86-fold increases in the ACE activities in rat carotid bodies subjected to 7-, 14-, 21-, and 28-day hypoxia, respectively, compared with results from controls (Table 1).

**Table 1. Regulation of ACE activities from normoxic and hypoxic carotid bodies during varying period of chronic hypoxia study**

<table>
<thead>
<tr>
<th>Days of Hypoxia</th>
<th>Normoxia</th>
<th>Hypoxia</th>
</tr>
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<tbody>
<tr>
<td>7</td>
<td>15.00±0.80</td>
<td>30.95±1.23*</td>
</tr>
<tr>
<td>14</td>
<td>8.73±1.38</td>
<td>30.25±1.42*</td>
</tr>
<tr>
<td>21</td>
<td>11.41±0.83</td>
<td>31.83±2.23*</td>
</tr>
<tr>
<td>28</td>
<td>13.18±3.45</td>
<td>24.53±3.64</td>
</tr>
</tbody>
</table>

Values are means ± SE. ACE, angiotensin-converting enzyme. *Statistical significance compared with the normoxic group (P<0.05).
**Statistical significance when compared with the normoxic group (P < 0.05). *Statistical significance when compared with activity in corresponding group without application of inhibitor (P < 0.05).**

**Fig. 1.** Effect of 7-day hypoxia on angiotensin-converting enzyme (ACE) activity in carotid bodies. C7, ACE activity in 7-day normoxic carotid bodies; H7, ACE activity in 7-day hypoxic carotid bodies; Lung, ACE activity in normoxic lung; C7-inhibitor, ACE activity with ACE inhibitor in 7-day control carotid bodies; H7-inhibitor, ACE activity with ACE inhibitor in 7-day hypoxic carotid bodies; L-inhibitor, ACE activity with ACE inhibitor in lung. ACE activity was expressed in nmol·µg⁻¹·min⁻¹ of 7-day normoxic and hypoxic carotid bodies. Data are expressed as means ± SE (n = 5 assays for each group). **Statistical significance when compared with the normoxic group (P < 0.05). *Statistical significance when compared with activity in corresponding group without application of inhibitor (P < 0.05).**

**DISCUSSION**

Hypoxia is known to be one of the pivotal factors that renders a regulatory effect on the expression and function of local RAS components from various tissues and organs. These include, to name a few, the lung (7, 40, 42, 56), the kidney (43), the heart (41), the epididymis (30), and the pancreas (6, 21). Of great importance is the recent evidence for the existence of an intrinsic RAS in the carotid body (26). The key components of such a local RAS are subject to the fine regulation by chronic hypoxia (17, 27, 32). In those studies, mRNA expression for individual components, such as the ANG II receptors (AT₁α, AT₁b, and AT₂-receptor subtypes), angiotensinogen, and ACE, was found to be present in the carotid body. Interestingly, mRNAs of angiotensinogen and ACE were expressed, whereas renin mRNA was absent in the carotid body, suggesting that a renin-independent angiotensin system might be operating in the carotid body (26). On the other hand, these RAS components such as AT₁ receptors (17, 32) and angiotensinogen (27) could be finely upregulated to different degrees after exposure to chronic hypoxia.

Notwithstanding the importance of such a local system in the carotid body, the regulation of ACE activity and its potential role in the production of ANG II during chronic hypoxia remain equivocal. In view of this fact, the present study has focused on ACE activity, ACE being a critical enzyme for the determination of the conversion of ANG I into physiologically active ANG II and the degradation of bradykinin in the carotid body during exposure to chronic hypoxia. Results showed that ACE activity exhibited a time-dependent increase during 7, 14, 21, and 28 days of chronic hypoxia. There was a significant increase in the ACE activity of rat carotid bodies under the stress of chronic hypoxia starting at day 7 and sustaining at day 14 to day 21 in the hypoxic groups. Afterward, however, this began to level off during further hypoxic treatment. Indeed, the increase in the 28-day hypoxic group was found to be stas
tically insignificant when compared with its respective normoxic controls. These results prompt us to suggest that enhanced activity of ACE could be responsible for the increased generation of ANG II in the carotid body from as early as day 7 to ~1-month of chronic hypoxia. The resultant level of ANG II together with upregulated expression of AT1 receptors may be physiologically important in the modulation of excitability of carotid chemoreceptor activity and thus the ventilatory drive during chronic hypoxia. However, the relative importance of ACE and importance of the alternative enzymes to renin as well as angiotensinogen in the carotid body, finally dictating the biosynthetic cascade of ANG II production, need further investigation.

The carotid body acts as the first gate for detecting the rapid changes in oxygen tension and composition in the arterial blood. Its local RAS components together with the activity of ACE were upregulated by chronic hypoxia. In this regard, there was a differential effect on the regulation of the ACE activity by chronic hypoxia, notably in various tissues or organs. Previous studies have shown that the serum ACE activity is reduced in human subjects at high altitude (35, 36). ACE activity decreases in the lung (23, 24, 45), whereas it increases in the kidney (45) and pancreas (22) of chronically hypoxic rats. Our results showed that there was an abrupt and significant effect on the ACE activity in the rat carotid bodies that underwent 7, 14, and 21 days of hypoxia, suggesting that the effect of chronic hypoxia on the ACE activity is organ specific. In this context, our previous study (27) has also shown an increase in the mRNA expression of ACE in the carotid body in hypoxia. Thus the elevated ACE activity may be due to an increased level of ACE protein in the carotid body during chronic hypoxia.

It is noted that the rat carotid bodies with 28-day hypoxia showed a statistically insignificant increase in the ACE activity. The adaptation and compensation by the carotid body might account for the change in ACE activity during chronic hypoxia. In fact, the increase in ACE activity is associated with an increased volume of the carotid body due to remodeling in vasculature, development of cellular hypertrophy, and increased mitotic activities in the chemosensitive cells during chronic hypoxia (25, 48, 52). It has been reported that elevation of ACE activity in pulmonary artery smooth muscle cells by hypoxia may play an important role in the development of hypoxic pulmonary hypertension (54). An increase in ANG II levels via the pathway of ANG II formation by ACE may also play an important role in the cardiac hypertrophy of hamster caused by the overloaded state (34). Therefore, the upregulation of ACE activity in rat carotid body by chronic hypoxia should be of physiological and clinical relevance, for example, for high-altitude physiology and in some clinical conditions of congenital heart defects and chronic lung disease (14, 15).

The local RAS in the carotid body might play a crucial role in the hemodynamic and pathophysiological changes in hypoxia. The increased ACE activity can elevate local production of ANG II, thus enhancing the excitability of the carotid chemoreceptor. In addition to the increase in the ventilatory drive during hypoxia, studies have shown that the activation of carotid chemoreceptor can cause sodium and water diuresis, thus leading to a reduction in plasma volume and an increase in hemococoncentration in early hypoxia (19). In addition, basal levels of and exercise-induced elevation in plasma renin activity and aldosterone are less in mountaineers (49, 55), high-altitude natives, and patients with chronic lung diseases (8), suggesting that hypoxia interferes with the circulating RAS. This downregulation may reduce the sodium and water retention that may occur at high altitude. On the other hand, the upregulation of the local RAS in the carotid body may be compensatory to the suppressive effect of hypoxia on the circulating RAS for the maintenance of sympathetic activities. The outcomes of these adaptive changes are centrally linked to the physical performance and development of pulmonary hypertension and acute mountain sickness at high altitude. Indeed, it was found that the levels of serum ACE activity and the insertion/deletion polymorphism of the ACE gene are associated with athletic performance and arterial oxygen saturation at high altitude (3, 18, 39, 53) and a number of cardiopulmonary diseases (1, 2, 9, 10, 38, 50). Thus changes of local ACE activity in the carotid body by prolonged hypoxia might be potentially important in high altitude and in athlete physiology, in addition to having pathophysiological relevance (31).

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