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Effects of angiotensin-converting enzyme and angiotensin Ⅱ on hypoxia-induced proliferation of cultured intra-pulmonary artery smooth muscle cells

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KEY WORDS angiotensin-converting enzyme；angiotensin Ⅱ；pulmonary artery；cultured cells；vascular smooth muscle；cell hypoxia

ABSTRACT

AIM：To investigate whether local angiotensin-converting enzyme (ACE) and endogenous angiotensin Ⅱ (ANGⅡ) are involved directly in the proliferation of intra-pulmonary artery smooth muscle cells (PASMC) induced by hypoxia. **METHODS**：Smooth muscle cells isolated from rabbit intra-pulmonary artery (300 – 400 μm-diameter) were cultured and used in the 3 – 8 passages. [^{3}H] Thymidine incorporation and cell counts were used to measure PASMC proliferation. **RESULTS**：Exposure of PASMC to hypoxia for 24 h resulted in an increase in the [^{3}H] thymidine incorporation and cell number by 166.6 % and 52.0 % as compared with normoxia ($P < 0.01$). Treatment with either captopril or losartan markedly inhibited the increase，compared with the control，[^{3}H] thymidine incorporation was inhibited by 51.3 % ($P < 0.01$) and 49.8 % ($P < 0.01$) and cell number was inhibited by 22.2 % ($P < 0.01$) and 17.9 % ($P < 0.01$)，respectively，while PD-123319 showed no significant effect. **CONCLUSION**：Local overexpression of PASMC ACE and ANGⅡ play an important role in the proliferation of PASMC induced by hypoxia.

INTRODUCTION

Hypoxia results in a continued stimulus to pulmonary vasoconstriction with consequent vascular remodeling，which is characterized by hyperplasia and hypertrophy of intra-pulmonary artery smooth muscle cells (PASMC)[1]. But the mechanism governing this change is still unknown.

Recent research has demonstrated that the renin-angiotensin system (RAS) is not only a circulating hormonal system but also a tissue system，widespread in cardiovascular organs，which has been implicated in vascular remodeling accompanying various cardiovascular diseases[2]. But the role of RAS in pulmonary disease has received far less attention. Our previous study suggested that PASMC ACE activity and gene expression were increased by hypoxia[3]. It is well known that ANGⅡ，which is converted by ACE from ANGⅠ，can stimulate vascular smooth muscle cell proliferation and hypertrophy in addition to its traditional role as a vasoconstrictor.

Thus，RAS might play an important role in the pulmonary vascular remodeling of pulmonary hypertension induced by hypoxia. In this study，we used ACE inhibitor captopril，the ANGⅡ type 1 receptor (AT1) antagonist losartan and the ANGⅡ type 2 receptor (AT2) antagonist PD-123319 to investigate whether local ACE and endogenous ANGⅡ are involved directly in the proliferation induced by hypoxia of PASMC.

MATERIALS AND METHODS

Materials Captopril was purchased from Sigma Chemical Co，losartan and PD-123319 from DuPont Merck Pharmaceutical Co. Fetal bovine serum (FBS) was purchased from Hangzhou Si Ji Qing Co. Dulbecco's modified Eagle's medium (DMEM) was provided by Gibco and [^{3}H] thymidine by Shanghai Institute of Nuclear Research，Chinese Academy of Sciences.

Cell culture PASMC were isolated from 2 – 3 month old New Zealand strain rabbits (obtained from Animal Center of Hunan Medical University) as described previously[4]. The cells were characterized as smooth

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muscle based on their growth in "hill and valleys" and expression of smooth muscle-specific α-actin mRNA. Cells were grown at 37 ℃ under 5 % CO_2 in DMEM supplemented with benzylpenicillin 100 kU·L^{-1}, streptomycin 100 mg·L^{-1} and 10 % FBS. Experiments were performed with cells from passage 3 – 8.

Exposure of PASMC to hypoxia Chamber in which cells cultures could be incubated under hypoxia was made by us[3], with two nipple fittings on opposite sides, providing an inlet and outlet to set up mixed gas (3 % O_2 + 5 % CO_2 + 92 % N_2) circulation. After putting in the cells, the chamber was sealed and filled with mixed gas at the speed of 30 mL/min. After 4 – 5 h the P_{O_2} in the medium decreased to (2.98 ± 0.16) kPa while P_{CO_2} and pH did not change.

[^{3}H] Thymidine incorporation PASMC were seeded at 1 × 10^5 cells/well in 24 well plates. After a 72-h incubation in DMEM-10 % FBS (1 mL/well) to induce a quiescent state, the medium was replaced with fresh DMEM-1 % FBS containing [^{3}H] thymidine (37 kBq/well) and various drugs (captopril 10 μmol·L^{-1}, losartan 1 μmol·L^{-1}, or PD-123319 10 μmol·L^{-1}) for a total incubation period of 24 h under hypoxia or normoxia. Then the medium was removed, and PASMC were rinsed twice with cold PBS fixed with 2 % perchloric acid. After an additional rinse with PBS, the PASMC were trypsinized and resuspended in 10 % trichloroacetic acid (TCA) at 37 ℃ for 10 min to lyse the cells. The cell lysate was vacuum-filtered through a glass fiber fitter. Having been washed with 10 % TCA followed by 95 % ethanol, the filter was dried. The PASMC [^{3}H] thymidine incorporation into DNA was expressed as Bq with liquid scintillation counter.

Cell counting As mentioned above, the PASMC were incubated in DMEM-10 % FBS (1 mL/well) to induce a quiescent state, the medium was replaced with DMEM-1 % FBS containing captopril, losartan, or PD-123319. After a 24-h culture under hypoxia or normoxia, the PASMC were rinsed with PBS, trypsinized, and resuspended in 1 mL DMEM. The number of PASMC was determined by a hemocytometer.

Statistic analysis All values were expressed as $\bar{x} \pm s$ and assessed by t test. Measurements of each group were performed in triplicate.

RESULTS

Effect of hypoxia on the proliferation of PASMC Hypoxia (24 h) markedly stimulated the cultured PASMC proliferation. As compared with normoxia, the [^{3}H] thymidine incorporation and cell counts increased 166.6 % ($P < 0.01$) and 52.0 % ($P < 0.01$), respectively (Tab 1).

Tab 1. Effect of hypoxia on the proliferation of PASMC. $\bar{x} \pm s$ of 6 independent experiments. Average of duplicate determination. $^cP < 0.01$ *vs* hypoxia.

	[^{3}H] Thymidine incorporation (Bq/well)	Cell number (10^{-5} × cells/well)
Normoxia	299 ± 35	23.5 ± 1.5
Hypoxia	797 ± 79^c	35.8 ± 0.9^c

Effect of captopril, losartan, and PD-123319 on the proliferation of PASMC induced by hypoxia Captopril, losartan, and PD-123319 caused no change in the [^{3}H] thymidine incorporation and cell number of PASMC cultured in normoxia. Captopril and losartan markedly inhibited the proliferation induced by the 24-h hypoxia, their inhibition rates of [^{3}H] thymidine incorporation were 51.3 % and 49.8 %, and cell number 22.2 % and 17.9 %, respectively; Whereas PD-123319 revealed no effect on the proliferation induced by hypoxia (Tab 2).

Tab 2. Effect of captopril (Cap), losartan (Los), and PD-123319 (PD) on the proliferation of PASMC induced by hypoxia (H). $\bar{x} \pm s$ of 6 independent experiments. Average of duplicate determination. $^aP > 0.05$ *vs* normoxia (N). $^dP > 0.05$, $^fP < 0.01$ *vs* hypoxia.

	[^{3}H] Thymidine incorporation (Bq/well)	Cell number (10^{-5} × cells/well)
N	299 ± 35	23.5 ± 1.5
N + Cap	315 ± 24^a	22.4 ± 1.6^a
N + Los	313 ± 17^a	23.2 ± 1.1^a
N + PD	289 ± 42^a	24.2 ± 0.8^a
H	797 ± 79	35.8 ± 0.9
H + Cap	388 ± 38^f	27.2 ± 1.6^f
H Los	401 ± 22^f	28.7 ± 1.1^f
H + PD	773 ± 26^d	35.2 ± 2.2^d

DISCUSSION

Although ACE inhibitor and ANG Ⅱ antagonist could inhibit the development of pulmonary hypertension induced by hypoxia[5-7], some authors reported that lung

ACE activity was decreased by chronic hypoxia[8,9]. This paradox was difficult to explain. We think that whole lung ACE activity may not reflect local pulmonary vascular ACE expression. Nicholas *et al*[10] found that ACE expression was increased locally in the walls of small pulmonary arteries undergoing structural remodeling, despite there was a generalized reduction in whole pulmonary ACE activity. The measured reduction of whole ACE activity in the hypoxic lung was due to marked reduction in alveolar capillary ACE. Our previous study[3] also observed that ACE activity and gene expression increased in cultured small PASMC exposed to hypoxia. The results of this study indicate that exposure of cultured PASMC to hypoxia for 24 h could induce an increase in PASMC proliferation and ACE inhibitor (captopril) significantly reduced the elevation induced by hypoxia. These results are consistent with our previous report, suggesting that locally overexpressed ACE might play an important role in pulmonary hypertension.

ACE is the main enzyme that catalyzes the conversion of ANG Ⅰ to ANG Ⅱ. So the increase in local ACE might cause overproduction of ANG Ⅱ. Recent research works have asserted that ANG Ⅱ may contribute to the development of chronic hypoxic pulmonary hypertension via its vasoconstrictor action or via effects on migration and growth of vascular smooth muscle cells through AT1 receptor[7,11]. The present study supports this idea by finding that AT1 antagonist losartan obviously reduced cell number and [^{3}H] thymidine incorporation of PASMC induced by hypoxia, while no change was observed on PASMC treated with AT2 antagonist PD-123319.

However, it is still unclear from studies with ACE inhibitor whether these effects are solely due to suppression of ANG Ⅱ levels or these could be explained by changes in levels of other vasoactive substances, especially bradykinin, since ACE is also one of the main enzyme that hydrolyzes bradykinin. It has been widely reported that bradykinin is a potent vasodilator and an anti-growth factor. So the protective effect of ACE inhibitor may be partly due to bradykinin[12,13]. However, there is no evidence that bradykinin plays an important role in hypoxic pulmonary circulation. The different responses of the systemic and pulmonary circulation may come from the relative paucity of bradykinin receptors in pulmonary vascular tissue[14]. Although no work on bradykinin has been done in present study, yet our data points out that there is no difference between the rate of [^{3}H] thymidine incorporation and cell number inhibited by captopril and losartan, thus indirectly supporting the above mentioned concept.

In conclusion, the mechanism governing hypoxic pulmonary hypertension might be that hypoxia increases PASMC ACE activity and its gene expression, subsequently causing an overproduction of ANG Ⅱ which mediate the proliferation induced by hypoxia via AT1 receptor. We suggest that local and endogenous ANG Ⅱ may play an important role in the development of hypoxic pulmonary hypertension.

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血管紧张素转化酶和血管紧张素Ⅱ对低氧促培养的肺内小动脉平滑肌细胞增殖作用的影响

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关键词 血管紧张素转换酶；血管紧张素Ⅱ；肺动脉；培养的细胞；血管平滑肌；细胞低氧

目的：研究局部血管紧张素转化酶及血管紧张素Ⅱ和低氧促进肺动脉平滑肌细胞增殖作用之间的关系. 方法：分离培养肺内小动脉平滑肌细胞，测定[^{3}H] thymidine 掺入和细胞计数作为细胞增殖的指标. 结果：低氧显著促进培养的肺内小动脉平滑肌细胞增殖，其[^{3}H] thymidine 掺入和细胞计数分别增加 166.6 %（$P<0.01$）和 52.0 %（$P<0.01$）. captopril 和 losartan 预处理可显著抑制低氧对肺内小动脉平滑肌细胞增殖的促进作用，[^{3}H] thymidine 掺入分别被抑制 51.3 %（$P<0.01$）和 49.8 %（$P<0.01$），细胞计数分别被抑制 22.2 %（$P<0.01$）和 17.9 %（$P<0.01$）. 而 PD-123319 基本无明显作用. 结论：肺内小动脉平滑肌细胞局部血管紧张素转化酶的过度表达及血管紧张素Ⅱ在低氧促平滑肌细胞增殖中发挥重要作用.

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