

## Inhibitory effects of captopril on hypoxia-induced proliferation and collagen synthesis in pulmonary vascular smooth muscle cells

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**KEY WORDS** captopril; pulmonary artery; vascular smooth muscle; anoxia; cell division; collagen; calcium channels

### ABSTRACT

**AIM:** To study the effect of captopril (Cap) on hypoxia-induced proliferation and collagen synthesis in vascular smooth muscle cells (VSMC). **METHODS:** VSMC were isolated from rabbit pulmonary artery. Cultured VSMC were evaluated by incorporation of [<sup>3</sup>H]thymidine and [<sup>3</sup>H]proline, cell number, and intracellular calcium concentration ( $[Ca^{2+}]_i$ ). **RESULTS:** Pretreatment of pulmonary VSMC with Cap  $1 \mu\text{mol} \cdot \text{L}^{-1}$  blocked hypoxia-induced increase in cell number and incorporation of [<sup>3</sup>H]proline and [<sup>3</sup>H]thymidine, which were decreased 25%, 21%, and 36%, respectively, as compared with hypoxic control. It also inhibited the increase of intracellular  $Ca^{2+}$  concentration under hypoxic condition. Addition of nifedipine inhibited hypoxia-stimulated increase in the collagen, DNA synthesis, and  $[Ca^{2+}]_i$ . Bay-K-8644 increased cell number (35%), DNA (55%), collagen synthesis (36%), and  $[Ca^{2+}]_i$  (33%) in pulmonary VSMC, that was completely abolished by Cap  $1 \mu\text{mol} \cdot \text{L}^{-1}$ . **CONCLUSION:** Cap inhibited hypoxia-induced proliferation and collagen synthesis in VSMC.

### INTRODUCTION

Pulmonary hypertension is a manifestation of a

wide variety of cardiac and pulmonary diseases<sup>[1]</sup>. The increased pulmonary artery pressure may result from a rise in pulmonary vascular tone, hypertrophy, and hyperplasia of vascular smooth muscle cells (VSMC) and intimal cell proliferation. Chronic hypoxia is the major pathological factors associated with pulmonary hypertension. Angiotensin II (Ang II) may contribute to the development of chronic hypoxic pulmonary hypertension via its vasoconstrictor action or via effects on VSMC migration and growth<sup>[1-4]</sup>. Captopril (Cap), an angiotensin-converting enzyme (ACE) inhibitors (ACEI) has been extensively investigated in systemic hypertension and left ventricular (LV) failure and has been shown to induce regression LV hypertrophy in systemic hypertension and congestive heart failure<sup>[5]</sup>. Administration of ACEI, not only decreased the development of chronic hypoxic pulmonary hypertension but also reduced the pulmonary artery pressure and the degree of vascular remodeling (VSMC proliferation and collagen synthesis increased) in experimental or clinical pulmonary hypertension<sup>[1-4]</sup>. Cap may block the increase in  $[Ca^{2+}]_i$  induced by KCl, norepinephrine, and Ang II via a voltage-dependent  $Ca^{2+}$  channel of which function and specificity were altered in SHR VSMC<sup>[6]</sup>. However, Cap effects and its mechanism on VSMC proliferation and collagen synthesis to hypoxia-induced pulmonary vascular remodeling *in vivo* remain uncertain. This study was to investigate the role of Cap on hypoxia-induced VSMC proliferation and collagen synthesis, and the underlying mechanisms.

### MATERIALS AND METHODS

**Reagents** Cap, Fura 2-AM, nifedipine (Nif), and all culture reagents were purchased from Sigma Chemical Co, USA. Bay-K-8644 was the product

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from Bary Co. [ $^3\text{H}$ ]proline (special activity 440 TBq·mol $^{-1}$ ) and [ $^3\text{H}$ ]thymidine (special activity 810 TBq·mol $^{-1}$ ) were purchased from China Institute of Atomic Energy, Beijing.

**Cell culture** VSMC were isolated from New Zealand white rabbits ( $n = 12$ , 8-wk old) pulmonary artery and cultured over 8–10 passages<sup>[7]</sup>. The cells grew in 5 % CO $_2$  + 95 % air at 37 °C for 4–5 d. The culture medium was Dulbecco's modified Eagle's medium (DMEM) supplemented with 20 % fetal calf serum (FCS). VSMC were seeded ( $1 \times 10^4$  cells/well) in 24-well plates and cultivated in culture medium until confluent. The medium was replaced by a serum-free medium for 72 h. The cells were then stimulated again with 10 % FCS. After another 24-h cultivation, the drugs were added. After 1 h, cells were placed in an air-tight incubator where normal air was replaced by a gas mixture of 95 % N $_2$  + 5 % CO $_2$  for 24 h. The pO $_2$  in media reached a level of 1.3–2 kPa.

**Incorporations of [ $^3\text{H}$ ]thymidine and [ $^3\text{H}$ ]proline, and cell count** Cap effects on hypoxia-induced DNA and collagen synthesis in cultured VSMC were evaluated by incorporation of [ $^3\text{H}$ ]thymidine and [ $^3\text{H}$ ]proline into cells. Twenty-four hours after addition of [ $^3\text{H}$ ]thymidine and [ $^3\text{H}$ ]proline, cells were washed 3 times with phosphate-buffered saline (PBS) and lysed in 0.1 % sodium dodecyl sulfate/NaOH 0.1 mol·L $^{-1}$ . The radioactivity was determined by liquid scintillation counter (Beckman L3801, USA). The cells were

counted in hermacytometer after staining with Trypan blue.

**Measurement of intracellular calcium concentration ([Ca $^{2+}$ ] $_i$ )** The cells were washed 3 times at 37 °C with Krebs-Hanseleit, HEPES buffer (NaCl 141, KCl 5.6, CaCl $_2$  3, KH $_2$ PO $_4$  1.4, MgSO $_4$  1.4, and HEPES 20 mmol·L $^{-1}$ , pH 7.4). [Ca $^{2+}$ ] $_i$  was measured by a fluorescent spectrophotometer (Shimadzu RF5000, Japan) with  $\lambda_{ex}$  340 nm and  $\lambda_{em}$  380 nm. [Ca $^{2+}$ ] $_i$  change was described as the ratio of 340 nm/380 nm (F1/F2) and the minimum F1/F2 was indicated as 0 in every figure<sup>[7]</sup>.

**Statistics** Data were expressed as  $\bar{x} \pm s$  and analyzed with unpaired  $t$  test.

## RESULTS

### Proliferation in cultured pulmonary VSMC

Hypoxia increased the cell number (47 %) and [ $^3\text{H}$ ]thymidine incorporation (75 %). Cap 1  $\mu\text{mol} \cdot \text{L}^{-1}$  decreased cell number (25 %), [ $^3\text{H}$ ]thymidine incorporation (36 %) under hypoxia condition. However, treatment with Cap 1  $\mu\text{mol} \cdot \text{L}^{-1}$  under normal condition caused no significant change. Nif 1  $\mu\text{mol} \cdot \text{L}^{-1}$  markedly diminished the hypoxia-induced increase in the cell number and [ $^3\text{H}$ ]thymidine incorporation (Tab 1).

**Collagen synthesis in cultured pulmonary VSMC** Under hypoxic condition, collagen protein synthesis was increased 48 % as measured by

Tab 1. Effects of captopril 1  $\mu\text{mol} \cdot \text{L}^{-1}$  and nifedipine 1  $\mu\text{mol} \cdot \text{L}^{-1}$  on hypoxia- and Bay-K-8644 (1  $\mu\text{mol} \cdot \text{L}^{-1}$ )-induced proliferation, collagen synthesis, and Ca $^{2+}$  influxes in cultured pulmonary smooth muscle cells.  $n = 4$  wells for each group,  $1 \times 10^4$  cells/well.  $\bar{x} \pm s$ .  $^aP > 0.05$ ,  $^bP < 0.05$ ,  $^cP < 0.01$  vs control.  $^dP > 0.05$ ,  $^eP < 0.05$ ,  $^fP < 0.01$  vs hypoxia group.  $^gP > 0.05$ ,  $^hP < 0.05$ ,  $^iP < 0.01$  vs Bay-K-8644 group.

Group	Cell number (per well)	[ $^3\text{H}$ ]Thymidine/ kBq·mol $^{-1}$	[ $^3\text{H}$ ]Proline/ kBq·mol $^{-1}$	Ca $^{2+}$ /nmol·L $^{-1}$
Control	26 213 $\pm$ 2 540	795 $\pm$ 54	117 $\pm$ 14	221 $\pm$ 11
Hypoxia	38 623 $\pm$ 3 210 <sup>c</sup>	1 399 $\pm$ 113 <sup>c</sup>	173 $\pm$ 5 <sup>c</sup>	287 $\pm$ 21 <sup>c</sup>
Cap	27 854 $\pm$ 2 890 <sup>f</sup>	769 $\pm$ 78 <sup>f</sup>	126 $\pm$ 23 <sup>f</sup>	232 $\pm$ 23 <sup>f</sup>
Cap + Hypoxia	29 054 $\pm$ 4 460 <sup>f</sup>	882 $\pm$ 151 <sup>f</sup>	135 $\pm$ 27 <sup>f</sup>	241 $\pm$ 32 <sup>f</sup>
Nif	28 965 $\pm$ 2 031 <sup>f</sup>	862 $\pm$ 107 <sup>f</sup>	115 $\pm$ 14 <sup>f</sup>	218 $\pm$ 24 <sup>f</sup>
Nif + Hypoxia	28 780 $\pm$ 3 450 <sup>f</sup>	882 $\pm$ 98 <sup>f</sup>	129 $\pm$ 12 <sup>f</sup>	229 $\pm$ 22 <sup>f</sup>
Bay-K-8644	35 421 $\pm$ 5 430 <sup>c</sup>	1 230 $\pm$ 107 <sup>c</sup>	160 $\pm$ 18 <sup>c</sup>	296 $\pm$ 31 <sup>c</sup>
Bay-K-8644 + Cap	27 989 $\pm$ 2 450 <sup>f</sup>	843 $\pm$ 98 <sup>f</sup>	122 $\pm$ 23 <sup>f</sup>	235 $\pm$ 18 <sup>f</sup>

[ $^3\text{H}$ ]proline. Pretreatment of VSMC with Cap I  $\mu\text{mol}\cdot\text{L}^{-1}$  blocked hypoxia-induced increase in [ $^3\text{H}$ ]proline incorporation, which was decreased 22 % as compared with hypoxia control. Addition of Nif I  $\mu\text{mol}\cdot\text{L}^{-1}$  inhibited hypoxia-stimulated increase in the collagen synthesis (Tab I).

**L calcium channel in cultured pulmonary VSMC** Bay-K-8644  $0.1 \mu\text{mol}\cdot\text{L}^{-1}$  increased the synthesis of collagen (36 %) and DNA (55 %), and cell number (35 %), which were completely abolished by Cap I  $\mu\text{mol}\cdot\text{L}^{-1}$  (Tab I).

Resting  $[\text{Ca}^{2+}]_i$  was  $(221 \pm 11) \text{ nmol}\cdot\text{L}^{-1}$  in pulmonary VSMC. Hypoxia increased the  $[\text{Ca}^{2+}]_i$  30 %, which was diminished by Nif I  $\mu\text{mol}\cdot\text{L}^{-1}$  and Cap I  $\mu\text{mol}\cdot\text{L}^{-1}$ . Bay-K-8644  $0.1 \mu\text{mol}\cdot\text{L}^{-1}$  increased the  $[\text{Ca}^{2+}]_i$  34 %. Addition of Cap I  $\mu\text{mol}\cdot\text{L}^{-1}$  entirely blocked Bay-K-8644-stimulated increase in the  $[\text{Ca}^{2+}]_i$  (Tab I).

## DISCUSSION

The results of our present study led to the following major conclusions:

1) Hypoxia promoted proliferation and collagen synthesis in VSMC, which resulted in medial wall thickening of already muscular vessels and in peripheral extension of VSMC in previously nonmuscular vessels<sup>[1-4,9]</sup>. These data are available on the relative contribution of VSMC hyperplasia and/or hypertrophy to hypoxia-induced pulmonary vascular remodeling.

2) Locally formed Ang II appeared to play a role in hypoxia-induced pulmonary hypertension. ACEI with Cap completely blocked hypoxia-induced proliferation and collagen synthesis in cultured pulmonary VSMC, which were abolished by losartan, a type I Ang II receptor blocker but not by a type II Ang II receptor blocker PD123319 (to be published). Ang II promoted migration and proliferation of VSMC, and stimulated the synthesis of the extracellular matrix by VSMC<sup>[10-11]</sup>.

3) Cap may inhibit hypoxia-induced proliferation and collagen synthesis, which prevented and regressed pulmonary vascular remodeling. The inhibitory effect of Cap may involve completely the blockade of conversion of Ang I to Ang II across in VSMC and the increase of bradykinin<sup>[1-4]</sup>.

4) Cap had inhibitory effects on hypoxia condition

of voltage-operated calcium channel. Addition of Nif inhibited hypoxia-stimulated increase in collagen, DNA synthesis, cell number, and  $[\text{Ca}^{2+}]_i$ . It indicated that voltage-operated calcium channel was involved in pathological condition such as hypoxia-induced pulmonary hypertension. Bay-K-8644 increased collagen, DNA synthesis, cell number, and  $[\text{Ca}^{2+}]_i$  in VSMC, which were completely abolished by Cap.

In conclusion, Cap inhibited hypoxia-induced proliferation and collagen synthesis, which prevented and regressed pulmonary vascular remodeling. The mechanism may be associated with Cap inhibitory effects on hypoxic condition of voltage-operated calcium channel and ACE in pulmonary VSMC.

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卡托普利对缺氧诱导的肺动脉平滑肌细胞增殖和胶原合成的抑制作用

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关键词 卡托普利; 肺动脉; 血管平滑肌;

缺氧症; 细胞分裂; 胶原; 钙通道

目的: 观察卡托普利(Cap)抑制缺氧诱导的肺动脉平滑肌细胞(VSMC)增殖和胶原合成的作用. 方法: 采用细胞计数, [<sup>3</sup>H]脱氧胸苷, [<sup>3</sup>H]脯氨酸掺入和细胞内游离钙测定的方法. 结果: 卡托普利(Cap, 1 μmol·L<sup>-1</sup>)抑制缺氧诱导的 VSMC 中细胞数目, [<sup>3</sup>H]脱氧胸苷和 [<sup>3</sup>H]脯氨酸掺入及细胞内游离钙的增高, 较缺氧组分别降低了25%, 36%, 21%和16%. 硝苯吡啶也具有上述抑制作用. Bay-K-8644 促进 VSMC 中细胞数目, [<sup>3</sup>H]脱氧胸苷和 [<sup>3</sup>H]脯氨酸掺入及细胞内游离钙的增高, 分别增加35%, 55%, 36%, 34%, 这种作用可被 Cap 阻断. 结论: Cap 抑制缺氧诱导的肺动脉平滑肌细胞增殖和胶原合成, 这可能与阻断 L 型钙通道有关.

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