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# Bradykinin $B_2$ receptor antagonist icatibant reduces inhibitory effect of captopril on growth of cultured neonatal rat cardiomyocytes

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**KEY WORDS** proto-oncogenes; myocardium; captopril; angiotensin Ⅱ; icatibant; cultured cells; leucine; uridine

**AIM:** To study whether endogenous kinins are negative modulators in the growth of cardiomyocytes and their possible cellular and molecular mechanisms. **METHODS:** Cultured neonatal rat cardiomyocytes were used. Intracellular RNA and protein synthesis were measured by [$^3$H]uridine incorporation and [$^3$H]leucine incorporation, respectively. The expression level of proto-oncogene c-*myc*, c-*fos* mRNA was observed by Northern blotting. **RESULTS:** Exposure of cardiomyocytes to captopril (Cap, 100 $\mu mol \cdot L^{-1}$) for 48 h inhibited the rates of [$^3$H]Urd and [$^3$H]Leu incorporations by 25 % and 26 %, respectively, and for 2 h inhibited c-*myc*, c-*fos* mRNA expression by 75 % and 55 %, respectively. Treatment of angiotensin Ⅱ (Ang Ⅱ, 1 $\mu mol \cdot L^{-1}$) for 48 h significantly increased the rates of [$^3$H]Urd and [$^3$H]Leu incorporations and for 1 h induced c-*myc*, c-*fos* mRNA overexpression, which were reduced by pretreatment with Cap (100 $\mu mol \cdot L^{-1}$). Icatibant acetate (Hoe 140, a specific antagonist of bradykinin $B_2$ receptor) 0.1 - 10 $\mu mol \cdot L^{-1}$ blocked the effects of Cap in a concentration-dependent manner. **CONCLUSION:** Endogenous kinins exhibited a negative modulatory effect on growth of cardiomyocytes *via* BK $B_2$ receptor.

Angiotensin-converting enzyme inhibitors (ACEI) prevent and regress cardiac hypertrophy, which can be attributed to not only Ang Ⅱ reduction but also bradykinin (BK) potentiation. The antihypertrophic effect of ACEI was abolished by a selective BK $B_2$ receptor antagonist Hoe 140 (icatibant acetate, Ica)[1,2]. Kinins may contribute to the cardioprotective effect of ACEI. Captopril (Cap) inhibited cardiomyocyte growth independent of Ang Ⅱ[3,4], and the overexpression of c-*myc*, c-*fos* mRNA seemed to be associated with cell proliferation and hypertrophy[5,6]. So, in this study, we concentrated on 2 questions: Were endogenous kinins involved in the growth of cardiomyocyte as negative modulators? Did c-*myc* and c-*fos* expression regulation mediate their effects?

## MATERIALS AND METHODS

**Drugs and probes** Angiotensin Ⅱ, captopril, medium 199, and 199-2 were purchased from Sigma Chemical Co. Icatibant acetate (Hoe 140) (*D*-Arg [hyp$^3$, Thi$^5$, *D*-Tic$^7$, Oic$^8$] BK, peptide content 77.4 %) was a gift from Dr Klaus J WIRTH in Hoechst AG. [$^3$H]uridine and [$^3$H]leucine were from Shanghai Institute of Nuclear Research, Chinese Academy of Sciences. The c-*myc*, c-*fos* cDNA probes and 18S SSO probe were synthesized and provided by the Department of Biochemistry of Cincinnati University. [$^{32}$P]dCTP and [$^{32}$P]ATP were obtained from Beijing Yahui Biotechnology Co.

**Cell culture**[7] Briefly, ventricles from 1 - 3-d-old Sprague-Dawley rats (supplied by the Animal Center of Hu-nan Medical University, Grade Ⅱ, Certificate № 20-009) were minced and dissociated with 0.1 % trypsin at 37 ℃. After dispersed, cells were incubated in 100-mL culture dishes for 90 min, nonattached cells (mainly cardiomyocytes) were removed and seeded into 24-well culture plates ($2.5 \times 10^5$ cells per well). Cells were incubated in Medium 199 supplemented with 10 % fetal calf serum, and bromodeoxyuridine 0.1 $mmol \cdot L^{-1}$ to prevent nonmyocardial cells proliferation for 48 h, and then replaced with serum-free Medium 199-2 for 24 h before drugs treatment.

**RNA and protein synthesis** RNA and protein synthesis in cardiomyocytes were evaluated by incorporation of [$^3$H]uridine and [$^3$H]leucine, respectively. The medium was

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aspirated and replaced with M199-2 containing [$^3$H]uridine or [$^3$H]leucine 37 kBq·L$^{-1}$, and test drugs for 48 h. At the end of labeling, cells were washed 3 times with cold D-Hanks' solution, detached with 0.1 % trypsin and 0.02 % edetic acid at 37 ℃ for 5 min, then collected on GF/C filters, precipitated and washed with 10 % trichloroacetic acid and 70 % ethanol. Filters were dried and radioactivity (dpm) was measured by a liquid scintillation counter (Beckman LS 3801, USA).

**Northern blot analysis** Total cellular RNA was extracted by the guanidinium thiocyanate-phenol-chloroform method[8], and size fractionated by 1 % agarose gel electrophoresis then transferred to nylon membranes. Northern blot hybridizations were performed with the [$^{32}$P]labeled c-*myc* and c-*fos* cDNA probes or 18S SSO probe in hybridization solution (50 % formamide, 5 × SSPE, 5 × Denhart's solution, 0.1 % sodium dodecyl sulfate, salmon sperm DNA 100 mg·L$^{-1}$) at 42 ℃ overnight. After washing, the membranes were placed between X-ray films and intensifying screens for autoradiography at − 70 ℃, then hybridization signals were quantitated by densitometry (CS-930, Japan).

**Statistical analysis** Data were expressed as $\bar{x} \pm s$. One-way ANOVA with multiple comparison methods and $Q$ test were used.

## RESULTS

**RNA and protein synthesis** Cap 100 μmol·L$^{-1}$ inhibited [$^3$H]Urd and [$^3$H]Leu incorporations by 25 % and 26 %, respectively *vs* control. Ang Ⅱ 1 μmol·L$^{-1}$ increased the rates of [$^3$H]Urd and [$^3$H]Leu incorporations by 45 % and 47 %, respectively *vs* control. Pretreatment with Cap (100 μmol·L$^{-1}$) reduced the effect of Ang Ⅱ. Ica 0.1 − 10 μmol·L$^{-1}$ abolished the effects of Cap in a concentration-dependent manner (Tab 1).

**c-*myc* and c-*fos* proto-oncogenes expression** Cardiomyocytes were treated with Cap (100 μmol·L$^{-1}$) for 0.5, 1, and 2 h. Cap inhibited c-*myc* and c-*fos* mRNA expression in a time-dependent manner (Fig 1, Tab 2). In cardiomyocytes treated with Cap 100 μmol·L$^{-1}$ for 2 h, c-*myc* and c-*fos* mRNA expression were inhibited by 75 % and 55 % *vs* control, respectively. Treatment of Ang Ⅱ 1 μmol·L$^{-1}$

**Tab 1.** [$^3$H]Urd and [$^3$H]Leu incorporation in cultured neonatal rat cardiomyocytes. $\bar{x} \pm s$ of 8 independent experiments. Average of duplicates constitutes one determination. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* control. $^fP < 0.01$ *vs* Cap (100). $^iP < 0.01$ *vs* Ang Ⅱ.

| Drug/μmol·L$^{-1}$ | Incorporation/Bq per well [$^3$H]leucine | [$^3$H]uridine |
|---|---|---|
| Control | 256 ± 38 | 2 230 ± 339 |
| Cap (1) | 244 ± 42 | 2 159 ± 347 |
| Cap (10) | 231 ± 32 | 1 867 ± 264 |
| Cap (100) | 188 ± 14$^c$ | 1 671 ± 241$^b$ |
| Hoe (0.1) + Cap (100) | 180 ± 22 | 1 776 ± 197 |
| Hoe (1) + Cap (100) | 218 ± 20$^{bf}$ | 2 087 ± 320 |
| Hoe (10) + Cap (100) | 236 ± 35$^{af}$ | 2 299 ± 449$^{af}$ |
| Hoe (10) | 241 ± 32 | 2 322 ± 180 |
| Ang Ⅱ (1) | 377 ± 61$^b$ | 3 225 ± 333$^c$ |
| Cap (100) + Ang Ⅱ (1) | 306 ± 23$^{bi}$ | 2 751 ± 250$^{ai}$ |

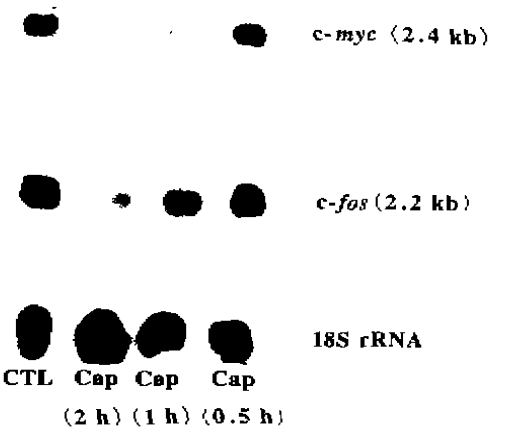


**Fig 1.** Autoradiography showing the effect of Cap (100 μmol·L$^{-1}$) for 0.5, 1, 2 h on c-*myc* and c-*fos* mRNA expression in cardiomyocytes.

**Tab 2.** Effect of Cap (100 μmol·L$^{-1}$) for 0.5, 1, 2 h on c-*myc* and c-*fos* mRNA expression in cardiomyocytes. The c-*myc* and c-*fos* mRNA/18S rRNA values of control group were arbitrarily set at 1.0 and the remaining groups were adjusted correspondingly. Data are $\bar{x} \pm s$ of 3 independent experiments. Average of triplicates constitutes one determination.
$^cP < 0.01$ *vs* control. $^fP < 0.01$ *vs* Cap for 2 h.

| Treatment | | Optical density ratio c-*myc* mRNA/18S rRNA | c-*fos* mRNA/18S rRNA |
|---|---|---|---|
| Control | (2 h) | 1.00 ± 0.09 | 1.00 ± 0.04 |
| Cap | (0.5 h) | 0.86 ± 0.06 | 0.99 ± 0.06 |
| | (1 h) | 0.50 ± 0.10$^{af}$ | 0.68 ± 0.10 |
| | (2 h) | 0.25 ± 0.07$^c$ | 0.45 ± 0.13 |

for 1 h markedly stimulated c-*myc* and c-*fos* expression about 6 and 3 times higher than those of control. Pretreatment with Cap 100 μmol·L$^{-1}$ for 1 h reduced the effect of Ang Ⅱ. Ica 10 μmol·L$^{-1}$ alone did not affect these gene expressions, but abolished the down-regulation of c-*myc* and c-*fos* mRNA expression induced by Cap 100 μmol·L$^{-1}$ completely (Fig 2, Tab 3).

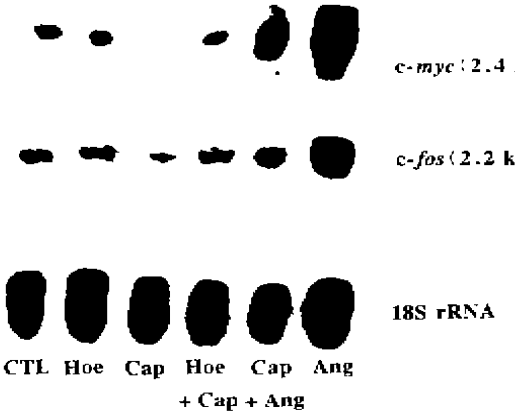


**Fig 2. Autoradiography showing c-*myc* and c-*fos* gene expressions in cardiomyocytes. CTL (control); Hoe: 10 μmol·L$^{-1}$ for 2 h; Cap: 100 μmol·L$^{-1}$ for 2 h; Hoe + Cap: pretreatment Hoe (10 μmol·L$^{-1}$) for 30 min following exposed to Cap (100 μmol·L$^{-1}$) for 2 h; Cap + Ang Ⅱ: pretreatment Cap (100 μmol·L$^{-1}$) for 1 h following Ang Ⅱ (1 μmol·L$^{-1}$) for 1 h.**

**Tab 3. Effect of icatibant acetate (Hoe 140) and Ang Ⅱ on captopril-induced c-*myc* and c-*fos* mRNA expession in cardiomyocytes. The c-*myc* and c-*fos* mRNA/18S rRNA values of control group were arbitrarily set at 1.0 and the remaining groups were adjusted correspondingly. Data are $\bar{x} \pm s$ of 3 independent experiments. Average of triplicates constitutes one determination. $^bP<0.05$, $^cP<0.01$ *vs* control. $^fP<0.01$ *vs* Cap for 2 h.**

| Treatment μmol·L$^{-1}$ | Optical density ratio c-*myc* mRNA/18S rRNA | c-*fos* mRNA/18S rRNA |
|---|---|---|
| Control | 1.00 ± 0.20 | 1.00 ± 0.08 |
| Ica (10) | 0.87 ± 0.17 | 0.96 ± 0.14 |
| Cap (100) | 0.21 ± 0.07$^b$ | 0.50 ± 0.04$^b$ |
| Ica (10) + Cap (100) | 0.95 ± 0.17 | 0.99 ± 0.06 |
| Cap (100) + Ang Ⅱ (1) | 3.85 ± 0.25$^{cf}$ | 2.9 ± 0.3$^{cf}$ |
| Ang Ⅱ (1) | 7.6 ± 0.5$^c$ | 5.1 ± 0.3$^c$ |

## DISCUSSION

Our results showed that Cap induced a growth inhibition in cardiomyocytes even in the presence of exogenous and high concentration of Ang Ⅱ, which is in agreement with the findings by Nagai *et al*[3] and Wang *et al*[4], suggesting that besides inhibition of Ang Ⅱ function, there exists other mechanism in growth inhibition of Cap. Cap reduce kinins inactivation, so as potentiation of kinins might be responsible for its effects. Nolly *et al*[9] ascertained that the heart contains an independent Kallikrein-kinin system. Minshall *et al*[10] found the presence of high-affinity BK $B_2$ receptors on cardiomyocytes. Chen *et al*[11] found that early Cap treatment in SHR rats inhibited the expression of c-*myc*, but did not change c-*fos* expression. Acute administration of benazepril or quinapril induced expression of c-*myc* and c-*fos* mRNA in the left ventricle[12]. The present study showed that Ang Ⅱ induced cardiomyocyte hypertrophy and c-*myc*, c-*fos* gene over-expressions; Cap inhibited cellular RNA and protein synthesis, and down-regulated c-*myc*, c-*fos* expression, which was abolished by Hoe 140, indicating that endogenous kinins, as negative regulatory factors, modulate the growth of cardiomyocytes and c-*myc*, c-*fos* expression *via* BK $B_2$ receptors. Addition of Cap to cardiomyocytes did not affect the generation of endogenous Ang Ⅱ, because BK antagonist blocked the Cap effect completely. It is possible that some Cap insensitive enzyme, such as chymase, may maintain Ang Ⅱ function. However, our study cannot exclude completely a non-specific sulfhydryl effect of Cap.

In summary, endogenous kinins exhibited a negative modulatory effect on growth of cardiomyocytes *via* BK $B_2$ receptor, and c-*myc* and c-*fos* proto-oncogenes mediated the cell growth regulation.

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## 缓激肽 $B_2$ 受体拮抗剂艾替班特降低卡托普利对培养新生大鼠心肌细胞生长的抑制

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**关键词** 原癌基因类；心肌；卡托普利；血管紧张素Ⅱ；艾替班特；培养的细胞；亮氨酸；尿嘧啶

**目的**：观察内源性激肽对培养新生大鼠心肌细胞生长的影响及其机制. **方法**：[$^3$H]尿嘧啶和[$^3$H]亮氨酸参入法检测 RNA 和蛋白质合成速率，Northern 杂交检测 c-*myc* 和 c-*fos* mRNA 表达. **结果**：卡托普利 100 μmol·L$^{-1}$孵育 48 h 显著抑制[$^3$H]尿嘧啶和[$^3$H]亮氨酸参入，孵育 2 h 明显抑制 c-*myc* 和 c-*fos* 基因表达. Ang Ⅱ 1 μmol·L$^{-1}$处理 48 h 刺激 RNA 和蛋白质合成，1 h 可上调 c-*myc* 和 c-*fos* 表达. Cap 100 μmol·L$^{-1}$部分抑制 Ang Ⅱ上述作用. 缓激肽 $B_2$ 受体拮抗剂 Ica (0.1 – 10 μmol·L$^{-1}$)剂量依赖性阻断 Cap 作用. **结论**：内源性激肽经 BK $B_2$ 受体介导对心肌细胞的生长起负调节作用.