

## Effects of verapamil on down-regulation of norepinephrine-induced $\beta$ adrenoceptors in cultured rat cardiomyocytes<sup>1</sup>

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**KEY WORDS** beta adrenergic receptors; verapamil; calcium; norepinephrine; myocardium; cultured cells; dihydroalprenolol; Fura-2

**AIM:** To determine whether verapamil (Ver) inhibits norepinephrine (NE)-induced  $\beta$  adrenoceptors down-regulation in cultured rat cardiomyocytes.

**METHODS:** [<sup>3</sup>H]-Dihydroalprenolol (DHA) radiobinding assay was used to measure  $\beta$  adrenoceptor density, fluorescent indicator Fura 2-AM was used to estimate levels of free cytosolic calcium ( $[Ca^{2+}]_i$ ).

**RESULTS:** Ver reduced  $[Ca^{2+}]_i$  and increased  $\beta$  adrenoceptor density, NE increased  $[Ca^{2+}]_i$  and reduced  $\beta$  adrenoceptor density of cultured cardiomyocytes, these effects were time and concentration dependent. Ver inhibited the above effects of NE. **CONCLUSIONS:** Ver increased  $\beta$  adrenoceptor density and inhibited NE-induced  $\beta$  adrenoceptor down-regulation of cardiomyocytes.

Long-term exposure of myocardial tissue to  $\beta$  adrenoceptor agonists reduces  $\beta$  adrenoceptor number and  $\beta$  adrenoceptor-mediated adenylate cyclase activity<sup>(1,2)</sup>. An inverse relation is also presented between the number of adrenoceptor and the level of NE in blood plasma of heart failure patients<sup>(3,4)</sup>. But the mechanisms underlying adrenoceptor down-regulation induced by agonists are incompletely understood.

Calcium-channel blockers, such as verapamil (Ver), are useful in treatment of several cardiovascular disorders. Hedberg<sup>(5)</sup> *et al* reported a 46% - 65% increase in the number of  $\beta$  adrenoceptors in human atria treated with calcium antagonist. Yonemochi<sup>(6)</sup> *et al* observed that  $\beta$  adrenoceptors of cultured rat

ventricular myocytes increased by 45% after 24 h of incubation with Ver  $1 \mu\text{mol} \cdot \text{L}^{-1}$ . But the mechanisms of this effect were still unclear. Some researchers<sup>(6,7)</sup> supposed that verapamil's ability to increase  $\beta$  adrenoceptor density was partly attributed to a decrease in  $[Ca^{2+}]_i$ . However, all of them did not directly measure exact level of intracellular calcium ion. The purpose of present study was to determine whether calcium antagonist Ver could increase  $\beta$  adrenoceptor density of normal cardiomyocytes and inhibit the NE-induced down-regulation of  $\beta$  adrenoceptors as well as their possible mechanism.

### MATERIALS AND METHODS

**Cell culture** Cardiomyocytes were prepared from neonatal rat ventricles. Hearts were removed from 2-3 d old neonatal SD rats of either sex under ether anesthesia. Ventricles were minced into 1-mm<sup>3</sup> pieces in phosphate-buffered solution (PBS). The pieces were washed 3 times with PBS, incubated with 0.06% collagenase (Type IV) for 5 min in 37 °C water bath with shaking. The supernatant containing erythrocytes and cell debris was discarded, the remaining pieces were treated with above method for 3-5 times. The free-floating cardiomyocytes were stored in Dulbecco's modified Eagle's medium (DMEM), centrifuged at  $500 \times g$  for 5 min to collect the isolated cardiomyocytes. The collected cardiomyocytes were seeded in glass culture bottles (Shanghai, China), and incubated for 90 min at 37 °C in 5% CO<sub>2</sub>. The fibroblasts attached to the bottom of the culture bottles after 1-2 h, and the floating cardiomyocytes were collected by decantation. The cell numbers were adjusted to  $2 \times 10^9$  cells/L in DMEM supplemented with 5% fetal bovine serum, benzylpenicillin  $1 \times 10^5 \text{ U} \cdot \text{L}^{-1}$  and streptomycin  $100 \text{ mg} \cdot \text{L}^{-1}$ . Cell suspension 10 mL was inoculated into a 25-mL glass culture bottle. After 48 h culture, more than 90% of the cells adhered to the bottom. Thereafter, the cultures were refed daily.

**Measurement of  $\beta$  adrenoceptors** The cardiomyocytes were scraped off with a rubber policeman to make a cell suspension, incubated with  $100 \mu\text{L}$  [<sup>3</sup>H]dihydroalprenolol ([<sup>3</sup>H]DHA, 5-100  $\mu\text{mol} \cdot \text{L}^{-1}$ ) for 12 h at 4 °C. A threefold volume of ice-cold Tris MgCl<sub>2</sub> buffer was then added to stop binding, the samples were collected on the F49 filter papers (Xinhua, Shanghai) through a filter. The filter papers were

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then dried for 20 min at 80 °C. The radioactivity was counted with Packard 4000 scintillator. Nonspecific binding was defined by using propranolol 100  $\mu\text{mol}\cdot\text{L}^{-1}$ .

**Measurement of intracellular calcium** Free cytosolic calcium was estimated with the fluorescent dye Fura 2-AM. Cardiomyocytes 2 mL were loaded with Fura 2-AM 5  $\mu\text{mol}\cdot\text{L}^{-1}$  for 35–40 min at 37 °C. BSA-Hanks' solution 2 mL was then added. The samples were centrifuged (500  $\times$  g) to collect the cardiomyocytes. The cardiomyocytes were incubated in 0.2 % BSA-Hanks' solution and then counted. Fura-2- $\text{Ca}^{2+}$  fluorescence ( $F$ ) was measured using an F-3000 spectrophotometer (Hitachi, Japan) with  $\lambda_{\text{ex}}$  340 nm and  $\lambda_{\text{em}}$  540 nm. Addition of 0.1 % triton-100, Fura-2- $\text{Ca}^{2+}$  maximal fluorescence ( $F_{\text{max}}$ ) was measured. Then, by addition of  $\text{MnCl}_2$  2  $\mu\text{mol}\cdot\text{L}^{-1}$  to extinguish fluorescence,  $F_{\text{min}}$  was measured. Free calcium concentration was calculated by the equation  $F = K_d(F - F_{\text{min}})/(F_{\text{max}} - F)$ .

**Protocol** The experiments were done after cardiomyocytes cultured for 4 d. The whole experiments were divided into 3 groups. First group, the different concentrations of NE or Ver were added into cultured bottles to incubate for 24 h to determine the concentration-response curves of NE and Ver on  $\beta$  adrenoceptors and  $[\text{Ca}^{2+}]_i$ . Second group, Ver 1  $\mu\text{mol}\cdot\text{L}^{-1}$  or NE 10  $\mu\text{mol}\cdot\text{L}^{-1}$  were added into cultured bottles to incubate for different time to determine the time-response curves. Third group, cardiomyocytes were cultured with NE 10  $\mu\text{mol}\cdot\text{L}^{-1}$  for different time to induce  $\beta$  adrenoceptor down-regulation, Ver 1  $\mu\text{mol}\cdot\text{L}^{-1}$  was added first, 30 min before adding NE to determine whether Ver can inhibit NE-induced  $\beta$  adrenoceptor down-regulation.

**Animals and reagents** Neonatal SD rats were purchased from the Experimental Animal Center of Railway Ministry. Ver, NE, Fura 2-AM, collagenase type IV, and propranolol were purchased from Sigma Chemical Co. [ $^3\text{H}$ ]DHA (specific activity 1.55 PBq  $\cdot$  mol $^{-1}$ ) was obtained from Beijing Nuclear Research Institute of China.

**Statistical analysis** Data were expressed as  $\bar{x} \pm s$  and analyzed with the paired  $t$  test.

## RESULTS

A concentration-dependent increase of  $\beta$  adrenoceptors and a concentration-dependent decrease of  $[\text{Ca}^{2+}]_i$  were observed after cardiomyocytes were incubated with Ver for 24 h (Tab 1).

A time-dependent increase of  $\beta$  adrenoceptors and a time-dependent decrease of  $[\text{Ca}^{2+}]_i$  were seen after cardiomyocytes were incubated with Ver 1  $\mu\text{mol}\cdot\text{L}^{-1}$ . There was a time-relation between  $[\text{Ca}^{2+}]_i$  decrease and  $\beta$  adrenoceptor increase, with  $[\text{Ca}^{2+}]_i$  decreasing first and  $\beta$  adrenoceptors increasing later (Tab 2).

**Tab 1. Intracellular  $\text{Ca}^{2+}$  and  $\beta$  adrenoceptors after incubation with Ver for 24 h.  $n = 6$  rats,  $\bar{x} \pm s$ .**

$^c P < 0.01$  vs control.

Verapamil/ $\text{nmol}\cdot\text{L}^{-1}$	$[\text{Ca}^{2+}]_i$ / $\text{nmol}\cdot\text{L}^{-1}$	$\beta$ Adrenoceptors, $\text{pmol/g protein}$
0	117 $\pm$ 10	139 $\pm$ 15
0.05	115 $\pm$ 10 <sup>c</sup>	142 $\pm$ 15 <sup>c</sup>
0.1	110 $\pm$ 8 <sup>c</sup>	159 $\pm$ 16 <sup>c</sup>
0.5	104 $\pm$ 15 <sup>c</sup>	170 $\pm$ 16 <sup>c</sup>
1	99 $\pm$ 7 <sup>c</sup>	196 $\pm$ 17 <sup>c</sup>

**Tab 2. Intracellular  $\text{Ca}^{2+}$  and  $\beta$  adrenoceptors after incubation with Ver 1  $\mu\text{mol}\cdot\text{L}^{-1}$ .  $n = 6$  rats,  $\bar{x} \pm s$ .**

$^c P < 0.01$  vs control.

Incubation time	$[\text{Ca}^{2+}]_i$ / $\text{nmol}\cdot\text{L}^{-1}$	$\beta$ Adrenoceptors, $\text{pmol/g protein}$
5 min (control)	120 $\pm$ 9	
10 min	109 $\pm$ 9 <sup>c</sup>	
30 min	99 $\pm$ 8 <sup>c</sup>	
60 min	99 $\pm$ 8 <sup>c</sup>	
2 h (control)		136 $\pm$ 14
4 h		147 $\pm$ 15 <sup>c</sup>
12 h		166 $\pm$ 15 <sup>c</sup>
24 h		186 $\pm$ 17 <sup>c</sup>
48 h		193 $\pm$ 17 <sup>c</sup>

A time-dependent increase of  $[\text{Ca}^{2+}]_i$  and a time-dependent decrease of  $\beta$  adrenoceptors were noted after cardiomyocytes were incubated with NE 10  $\mu\text{mol}\cdot\text{L}^{-1}$ . There was also a time-relation between  $[\text{Ca}^{2+}]_i$  increase and  $\beta$  adrenoceptor decrease, with  $[\text{Ca}^{2+}]_i$  increasing first and  $\beta$  adrenoceptor reducing later. If Ver was added beforehand, the effects of reducing  $\beta$  adrenoceptor density induced by NE was inhibited, but the number of  $\beta$  adrenoceptor was still lower. Ver almost completely prevented the increase of  $[\text{Ca}^{2+}]_i$  induced by NE (Tab 3).

## DISCUSSION

Our results showed that Ver induced increase of  $\beta$  adrenoceptor density and decrease of intracellular calcium ion both in time- and concentration-dependent manner. There was a time-relation presented between the change of intracellular calcium ion and that of  $\beta$  adrenoceptors, with intracellular free calcium reducing first and  $\beta$  adrenoceptors increasing later. These results suggested that the effect of increase  $\beta$

**Tab 3. Effects of Ver  $1 \mu\text{mol}\cdot\text{L}^{-1}$  on NE  $10 \mu\text{mol}\cdot\text{L}^{-1}$  induced intracellular  $\text{Ca}^{2+}$  increase and  $\beta$  adrenoceptor decrease.  $n = 6$  rats,  $\bar{x} \pm s$ .  $^aP > 0.05$ ,  $^bP < 0.05$ ,  $^cP < 0.01$  vs NE + Ver.**

Time	$[\text{Ca}^{2+}]_i / \text{nmol}\cdot\text{L}^{-1}$		$\beta$ Adrenoceptors, pmol/g protein	
	NE	NE + Ver	NE	NE + Ver
2 min	$144 \pm 14^b$	$121 \pm 12$		
5 min	$182 \pm 15^b$	$139 \pm 12$		
10 min	$256 \pm 19^c$	$158 \pm 15$		
30 min	$281 \pm 30^c$	$169 \pm 20$		
1 h			$132 \pm 15^a$	$135 \pm 15$
2 h			$110 \pm 14^a$	$116 \pm 15$
6 h			$50 \pm 20^c$	$76 \pm 19$
12 h			$25 \pm 10^c$	$63 \pm 19$

adrenoceptors of calcium antagonists might be attributed to the decrease of intracellular calcium ion.

The mechanisms of agonists-induced down-regulation of  $\beta$  adrenoceptors remain unclear. Some research<sup>[3,8]</sup> suggested that loss of receptors might be results of receptor internalization and degradation or the decrease of  $\beta$  adrenoceptor gene expression, but our present research found that NE-induced decrease of  $\beta$  adrenoceptors followed the significantly increase of intracellular  $[\text{Ca}^{2+}]_i$ . Moreover, it seems that there was a negative correlation presented between increase of intracellular  $[\text{Ca}^{2+}]_i$  and decrease of  $\beta$  adrenoceptors. Calcium antagonist Ver could inhibited the increase of intracellular  $[\text{Ca}^{2+}]_i$  of cardiomyocytes induced by NE, meanwhile, NE-induced reduction of  $\beta$  adrenoceptor density also partly reversed. These phenomena suggested that agonists-induced  $\beta$  adrenoceptor down-regulation might be partly caused by the increase of intracellular  $[\text{Ca}^{2+}]_i$ . Calcium antagonists Ver might have the effect of inhibiting NE-induced  $\beta$  adrenoceptor reduction and intracellular calcium ion increase.

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## 维拉帕米对去甲肾上腺素诱导的 培养大鼠心肌细胞 $\beta$ 受体下调的影响

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**关键词**  $\beta$  肾上腺素能受体; 维拉帕米; 钙; 去甲肾上腺素; 心肌; 培养的细胞; 双氢阿普洛尔; Fura-2

**目的:** 研究维拉帕米(Ver)是否能抑制去甲肾上腺素(NE)诱导的培养大鼠心肌细胞  $\beta$  受体下调及其可能机制。 **方法:**  $\beta$  受体密度用  $[^3\text{H}]$ -DNA 放射配基标记法, 细胞内游离钙用钙离子荧光探针 Fura 2-AM 法测定。 **结果:** Ver 能明显降低培养大鼠心肌细胞内游离钙水平, 增加  $\beta$  受体密度; NE 增加细胞内游离钙水平, 降低  $\beta$  受体密度; Ver 能明显抑制 NE 引起的细胞内游离钙增加和  $\beta$  受体密度的降低。 **结论:** Ver 能增加正常心肌细胞  $\beta$  受体密度, 抑制 NE 产生的心肌  $\beta$  受体下调。