Mechanism of \( \beta \)-Adrenergic Receptor Upregulation Induced by ACE Inhibition in Cultured Neonatal Rat Cardiac Myocytes

Roles of Bradykinin and Protein Kinase C

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Background—Although bradykinin is thought to contribute to the effects of ACE inhibitors on the cardiovascular system, its precise role remains to be elucidated. Evidence suggests that bradykinin might be important in the upregulation of \( \beta \)-adrenergic receptors (\( \beta \)-ARs) induced by ACE inhibitors, and the role of bradykinin in this effect has now been investigated with cultured neonatal rat cardiac myocytes.

Methods and Results—The density of \( \beta \)-ARs on the myocyte surface was determined with a binding assay with \([^{3}H]\)CGP-12177. Incubation of cultured myocytes for 24 hours with the ACE inhibitor captopril (1 \( \mu \)mol/L) increased \( \beta \)-AR density by 35% and enhanced the response of cells to isoproterenol but not to forskolin. Neither an angiotensin-II type 1 (AT\(_1\)) receptor antagonist, CV-11974, nor angiotensin-I affected \( \beta \)-AR density. However, the bradykinin B\(_2\) receptor antagonist Hoe 140 abolished the effect of captopril on \( \beta \)-AR upregulation in a dose-dependent manner. The protein kinase C inhibitor staurosporine (20 nmol/L) but neither indomethacin nor L-NAME also inhibited captopril-induced upregulation of \( \beta \)-ARs. Exogenous bradykinin increased the spontaneous beating frequency of cultured myocytes and Hoe 140 abolished this effect. Bradykinin level in the medium increased 1.4-fold by the treatment of cultured myocytes with captopril for 24 hours.

Conclusions—The results suggest that captopril enhances \( \beta \)-AR responsiveness by inducing \( \beta \)-AR upregulation and that the latter effect is mediated by activation of bradykinin B\(_2\) receptors and protein kinase C. These observations also offer insight into the different roles of ACE inhibitors and AT\(_1\) receptor antagonists in the treatment of heart failure. (Circulation. 1998;97:2268-2273.)

Key Words: bradykinin receptors, adrenergic, beta angiotensin myocytes

Inhibition of ACE improves cardiac function and prognosis in individuals with congestive heart failure. These beneficial effects have been thought to be attributable to inhibition of Ang-II formations, although ACE inhibitors also increase the concentration of bradykinin by inhibiting its degradation. Development of bradykinin B\(_2\)-receptor antagonist Hoe 140 has made it possible to investigate the role of an increase in bradykinin concentration by ACE inhibition under both physiologic and pathophysiologic conditions. Such studies have indicated that certain beneficial cardiac effects of ACE inhibitors, including protection of the heart from ischemia, prevention of the development of cardiac hypertrophy, preservation of cardiac function during long-term hypothermic storage of the heart, and improvement of cardiac function, may be due to bradykinin potentiation rather than to inhibition of Ang-II action. However, the precise mechanisms of these beneficial effects of bradykinin on the heart remain unknown.

Some studies have suggested that upregulation of \( \beta \)-ARs may contribute to the improvement of cardiac function that results from ACE inhibition. Furthermore, a role for bradykinin in upregulation of \( \beta \)-ARs induced by ACE inhibitors was suggested by our recent demonstration that an ACE inhibitor but not an Ang-II AT\(_1\) receptor antagonist increased \( \beta \)-AR density and enhanced the response to isoproterenol in cultured neonatal rat cardiac myocytes. In addition, the possibility that bradykinin acts in an autocrine or paracrine manner has been suggested by the detection of functional bradykinin B\(_2\) receptors as well as a local kallikrein-kinin system in cultured neonatal rat cardiac myocytes. On the basis of these observations, we hypothesized that bradykinin-induced upregulation of \( \beta \)-ARs contributes to the improvement in cardiac function attributable to ACE inhibitors.
To test this hypothesis, we have investigated whether Hoe 140 inhibits the effects of the ACE inhibitor captopril on the β-AR-adenylyl cyclase system. Moreover, because bradykinin also increases the synthesis of NO and prostaglandins as well as activates PKC in various tissues, the intracellular signaling pathway by which captopril induces β-AR upregulation was investigated with the use of Nω-nitro-ω-arginine methyl ester (L-NAME) (an inhibitor of NO synthase), indomethacin (an inhibitor of cyclooxygenase), and staurosporine (an inhibitor of PKC).

**Methods**

**Cell Culture**

Cardiac myocytes were prepared from neonatal rat ventricles by the modified Boloon’s method, as described previously. Briefly, hearts were removed from 3- to 5-day-old Wistar rats of either sex under ether anesthesia, and the ventricles were minced into 1-mm³ pieces in PBS. The pieces were washed three times with PBS, incubated in 0.02% EDTA for 5 minutes at 37°C with shaking, and centrifuged at 500g for 2 minutes to remove EDTA. The pieces were then incubated in 5 mL of Hanks’ balanced salt solution (HBSS) containing collagenase (2 mg/mL) (type IV; Cooper Biochemical) for 10 minutes in a 37°C water bath shaken at 120 rpm.

The supernatant containing erythrocytes and cell debris was discarded, and the remaining pieces of tissue were treated with dispase (1000 IU/mL) (Godo Shusei) in 5 mL of Ca²⁺- and Mg²⁺-free HBSS, with stirring, for 20 minutes at 37°C. Dispase treatment was repeated two more times, after which the free-floating cardiac myocytes were stored in Dulbecco’s modified Eagle’s medium (DMEM) and centrifuged at 500g for 2 minutes. The isolated cells were resuspended in DMEM, seeded in plastic culture dishes, and incubated at 37°C under 5% CO₂ in air for 90 minutes to form monolayers. The mean of the beating frequency in four independent cultures, the myocytes beat synchronously and at a constant frequency. Cells were used for the experiments on the 3rd to 5th days of culture, and spontaneous beating was monitored at 37°C for 15 minutes.

**Measurement of Cardiac β-AR Density**

Cardiac cell-surface β-ARs were identified according to the method of Limas and Limas by binding of the hydrophilic radioligand ([H]CGP-12177 (specific activity, 1.55 TBq/mmol; Amersham/Searle). On the 4th day of culture, myocytes were washed three times with assay buffer (0.25 mol/L sucrose, 10 mmol/L MgCl₂, and 50 mmol/L Tris-HCl, pH 7.4) and scrapped from the bottom of the dish with a rubber scraper. The detached cells were incubated for 16 hours at 4°C with 1 mL assay buffer containing [H]CGP-12177 at concentrations of 0.25 to 15 nmol/L, after which 3 mL of ice-cold assay buffer was added to each incubation and the samples were applied to GF/C filters (Whatman). The filters were washed three times with 6 mL ice-cold assay buffer, and the associated radioactivity was determined by scintillation spectroscopy in 5 mL of Aquasol-2 (New England Nuclear).

All measurements were performed in duplicate. The maximal number of binding sites (Bₘₐₓ) and the dissociation constant (Kᵦ) were calculated by Scatchard linear regression analysis, with r² > 0.90 as a criterion for acceptability of the data. Nonspecific binding was defined as binding in the presence of 10 μmol/L d,l-propranolol and was <20% of total binding at 5 nmol/L [H]CGP-12177.

The effects of captopril, Ang-I, and the Ang-II AT₁ receptor antagonist CV-11974 on β-AR density were evaluated by exposing the cells to each agent for 24 hours, before rinsing and incubating with 10 nmol/L [H]CGP-12177 as described above.

**Measurement of Bradykinin Concentration**

Bradykinin concentrations in cardiac myocyte culture medium were measured by radioimmunoassay capable of detecting the picogram per milliliter order of bradykinin. Two-milliliter aliquots of culture medium were collected from culture dishes after 24 hours of treatment with or without captopril and then transferred to a syringe containing 2 mL of solution with inhibitors (6000 kIU/mL aprotinin, 2 mg/mL soybean trypsin inhibitor, 10 mg/mL prostate sulfite, and 20 mg/mL EDTA-2Na).

**Drugs and Solution**

Captopril, CV-11974, and Hoe 140 were kindly provided by Sankyo Pharmaceutical Company, Takeda Pharmaceutical Company, and Hoechst Pharmaceutical & Chemicals K.K., respectively. Ang-I, L-NAME, indomethacin, staurosporine, and forskolin were obtained from Sigma. Captopril, Hoe 140, Ang-I, and L-NAME were dissolved in distilled water. CV-11974 was dissolved in 25 mol/L Na₂CO₃. Indomethacin and staurosporine were dissolved in 95% (vol/vol) ethanol. Forskolin was dissolved in 50% (vol/vol) dimethylsulfoxide (DMSO). The final concentrations of Na₂CO₃ (<25 mmol/L), ethanol (~0.09%), and DMSO (<0.1%) had no effect on the spontaneous beating or β-AR density of the cultured myocytes.

**Statistical Analysis**

Data are expressed as mean±SEM and were analyzed by one-way ANOVA and either Scheffe’s F test or Fisher’s protected least significant difference. A level of P<0.05 was considered statistically significant.

**Results**

**Effects of Captopril, Ang-I, and CV-11974 on the β-AR Density of Cultured Neonatal Rat Cardiac Myocytes**

Binding of [H]CGP-12177 to cultured myocytes saturated at a concentration of 10 nmol/L and Scatchard analysis revealed...
a single class of binding sites with a $B_{	ext{max}}$ of 104±10 fmol/mg protein and $K_D$ of 3.3±0.5 nmol/L (Figure 1). Prior incubation of myocytes with 1 µmol/L captopril for 24 hours increased the $B_{	ext{max}}$ by 35% but had no effect on the affinity for the ligand. Subsequent determinations of β-AR density were therefore performed in the presence of 10 nmol/L [3H]CGP-12177.

To clarify the mechanism of captopril-induced upregulation of β-ARs, we investigated the effects of Ang-I and the Ang-II AT1 receptor antagonist CV-11974 on β-AR density. In contrast to the effect of captopril, treatment of cells for 24 hours with either 1 µmol/L CV-11974 or 1 µmol/L Ang-I had no effect on β-AR density (Figure 2).

These results suggested that captopril-induced β-AR upregulation may be mediated by bradykinin.

**Functional Bradykinin B2 Receptor of Cultured Neonatal Rat Cardiac Myocytes and Bradykinin Levels in Mediums Conditioned by Treatment of Myocytes With Captopril**

Our previous studies revealed that both AT1 receptor and β-AR signal transduction pathways are functional in cultured neonatal rat cardiac myocytes, as assessed by the effects of receptor agonists or antagonists on beating frequency. However, it has remained unclear whether bradykinin B2 receptor signaling is operative in these cells.

**Effects of exogenous bradykinin on the spontaneous beating frequency of cultured cardiac myocytes.** A, Dose-response relation. Myocytes were exposed to various concentrations of bradykinin for 15 minutes. Data are mean±SEM of five separate experiments and are expressed as percentage of baseline. *P<0.05 vs baseline. B, Time course of the effects of 0.1 µmol/L bradykinin in the absence ( ) or presence (▲) of 10 µmol/L Hoe 140. Data are mean±SEM and are expressed as a percentage of baseline values. *P<0.05 vs baseline. Baseline beating frequency did not differ significantly among groups.

We therefore examined the effect of exogenous bradykinin and Hoe 140 on spontaneous beating frequency. Bradykinin increased the spontaneous beating frequency of myocytes in a dose-dependent manner (Figure 3A); the maximal increase from a control value of 76±4 bpm was 60%, apparent at 10 µmol/L bradykinin. The effect of 0.1 µmol/L bradykinin on spontaneous beating frequency was time dependent (Figure 3B). The increase in beating frequency reached a plateau of 22% after 5 minutes of exposure to bradykinin and sustained until 15 minutes. Furthermore, 10 µmol/L Hoe 140 reduced bradykinin-induced increase in spontaneous beating frequency from 24% to 8% after 15 minutes of exposure to bradykinin. Hoe 140 itself had no effect on beating frequency (data not shown). Thus bradykinin B2 receptor appeared to be functional in the cultured myocytes.

To directly prove the presence of bradykinin in cardiac myocytes culture mediums, we measured the bradykinin concentrations by radioimmunoassay. Bradykinin levels were 1.4-fold high in medium conditioned by 24 hours of treatment with captopril compared with that in untreated medium; however, it did not reach statistical significance (16.8±1.9 versus 11.7±1.2 pg/mL, $P=0.056$, $n=6$ to 7 from 6 separate cultures).

**Effects of Hoe 140 on Captopril-Induced β-AR Upregulation and Enhancement of the Response to Isoproterenol**

To investigate the possible role of endogenous bradykinin in captopril-induced upregulation of β-ARs, we examined the...
The response of beating frequency to captopril treatment, response to forskolin, and the enhancement of the response to isoproterenol induced by captopril were investigated with the use of L-NAME, indomethacin, and staurosporine, respectively. Cotreatment with neither L-NAME (10 μmol/L) nor indomethacin (10 μmol/L) affected β-AR upregulation induced by captopril (Figure 6). In contrast, cotreatment of cells with staurosporine at a concentration of 20 nmol/L prevented the captopril-induced increase in β-AR density.

Discussion

The major observations of the present study are as follows: (1) Exogenous bradykinin increased the spontaneous beating frequency of cultured myocytes in a dose-dependent and Hoe 140-sensitive manner. (2) The presence of bradykinin in cultured medium conditioned by treating myocytes with captopril was confirmed. (3) Hoe 140, a bradykinin B₂ receptor antagonist, abolished both the upregulation of β-ARs and the enhancement of the response to isoproterenol induced by captopril in cultured neonatal rat cardiac myocytes. And, (4) the PKC inhibitor staurosporine also inhibited the captopril-induced increase in β-AR density in these cells. These results suggest that the ACE inhibitor enhances the response to isoproterenol by upregulating β-ARS and that this effect is mediated by stimulation of B₂ receptors by endogenous bradykinin and activation of PKC.

Action of Bradykinin in an Autocrine and/or Paracrine Manner in Cultured Neonatal Rat Cardiac Myocytes

The existence of functional bradykinin B₂ receptors on cultured neonatal rat cardiac myocytes was confirmed by the present results showing that exogenous bradykinin in-
creased the frequency of spontaneous beating of these cells and that Hoe 140 abolished this effect. Furthermore, Nolly et al. had demonstrated the presence of a tissue kallikrein system in rat heart, which both contains and releases kallikrein and kininogen. In fact, we directly confirmed the presence of bradykinin in the medium of cultured neonatal rat cardiac myocytes. Bradykinin level (11.7 pg/mL) was converted to 11.0 fmol/g myocytes was not significantly different from 13 fmol/g wet heart weight reported by Campbell et al., although the captopril-induced increase of bradykinin level was low compared with previous report (1.4-fold versus 2- to 3-fold). Thus it appears likely that bradykinin acts in an autocrine and paracrine manner in this cultured system, although we cannot exclude the possibility that bradykinin or kininogen originates from contaminating cells in the myocyte cultures.

Role of Bradykinin in ACE Inhibitor–Induced Upregulation and Enhancement of the Response to Isoproterenol

It has been proposed that ACE inhibitors increase β-AR responsiveness by upregulating or inhibiting the downregulation of receptors as a result of reduced Ang-II stimulation of sympathetic nerve terminals and a consequent decrease in extracellular norepinephrine concentrations. However, a role for bradykinin in ACE inhibitor–induced improvement in cardiac function has been suggested by recent studies, including our demonstration that an ACE inhibitor but not an AT1 receptor antagonist increased β-AR density and the response to isoproterenol directly and independent of adrenergic activity in cultured neonatal rat cardiac myocytes. In addition, the action of captopril on β-adrenergic receptors appeared to be ACE-dependent effects, because a different class of ACE inhibitor, CV-3480, without a sulfhydryl moiety, also increased β-AR density. We have now shown that bradykinin B2 receptors mediate both β-AR upregulation and the increase in the response to isoproterenol induced by an ACE inhibitor.

Signal Transduction Pathways That Underlie the β-AR Upregulation Induced by Stimulation of Bradykinin B2 Receptors

Activation of bradykinin B2 receptors has been shown to induce NO synthesis, prostaglandin formation, and PKC activation. To identify the signal transduction pathway that mediates captopril-induced β-AR upregulation, we investigated the effects of L-NAME, indomethacin, and staurosporine, respectively. Our results indicate that PKC contributes to upregulation of β-ARs induced by ACE inhibition.

PKC activation is important in regulation of many cardiac functions. In cultured neonatal rat cardiac myocytes, however, the effects of activated PKC on β-AR signaling remain unclear. Thus Reupcke et al. showed that treatment of such cells for 10 minutes with the PKC activator phorbol 12 myristate 13-acetate resulted in downregulation of β-ARs, despite an increase in the adenylyl cyclase response to isoprenaline. The reason for the apparent discrepancy with our data suggesting that PKC induces upregulation of β-ARs is unclear. However, it may be attributable to the many differences in experimental details between the two studies or to the activation of different PKC isoforms by the respective agonists.

Johnson and Mochly-Rosen showed that 6 of the at least 10 members of PKC family of enzymes are present in cultured neonatal rat cardiac myocytes. These researchers also showed that the ε-PKC isozyme appears to contribute to the spontaneous beating of cultured myocytes. Furthermore, differential activation of PKC isozymes in response to physiologically relevant agonists (endothelin-1, ATP, and phenylephrine) has been demonstrated in neonatal rat heart.

Recently, Rouet-Benzineb et al. showed the downregulation of PKC activity and Ca2+-dependent isoform expression in rabbits with heart failure. They also hypothesized that this PKC downregulation may participate in the development of compensation that appears in heart failure.

Thus our results along with above considerations lead us to speculate that bradykinin potentiation by ACE inhibition may restore this downregulation of PKC, which results in upregulation of β-ARs and ultimately contributes to the improvement of cardiac function in heart failure.

In the present study, however, the identification of the PKC isoform (or isoforms) that mediates β-AR upregulation as well as the mechanism of this effect remain to be determined. Furthermore, it must be mentioned that the results of this study may not be applicable to adult human heart because PKC pattern is different between them.

In conclusion, upregulation of β-ARs may contribute to the improvement in cardiac function in response to treatment with ACE inhibitors, and this effect appears to be mediated by stimulation of bradykinin B2 receptors and activation of PKC. These observations offer insight into the different roles of ACE inhibitors and AT1 receptor antagonists in the treatment of congestive heart failure.

References


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