Characterization of a Potentially Reversible Increase in $\beta$-Adrenergic Receptors in Isolated, Neonatal Rat Cardiac Myocytes with Impaired Energy Metabolism


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SUMMARY. Previous studies have reported that the numbers of $\beta$- and $\alpha$-adrenergic receptors increase in ischemic myocardium. In vivo studies have raised questions regarding the mechanisms involved in the adrenergic receptor alterations and the consequences of these alterations. The purpose of this study was to evaluate potential relationships among $\beta$-adrenergic receptor changes, high energy phosphate reduction, and severity of cell injury in cultured neonatal rat myocytes treated with metabolic inhibitors. The potential for reversal of the receptor changes was also addressed. Binding parameters were measured using $[^{125}]$iodocyanopindolol. After 4 hours incubation in potassium cyanide and 2-deoxyglucose, there was a 43% increase in $\beta$-adrenergic receptor number, 41% decrease in adenosine triphosphate, and minimal morphological change in myocytes. Twenty-four hours after removal of the inhibitors, myocytes exhibited a return to normal of the receptor number and adenosine triphosphate level. Iodoacetate treatment for up to 3 hours resulted in marked reduction in adenosine triphosphate and increasing severity of cell injury. The number of $\beta$-adrenergic receptors was unchanged at 1.2 hours, increased at 1.5–2 hours, and decreased at 3 hours. Thus: (1) $\beta$-adrenergic receptor density increases during relatively early stages of injury in metabolically impaired myocytes with reduced adenosine triphosphate levels and decreases subsequently, after the myocytes become irreversibly injured; (2) the increased $\beta$-adrenergic receptor density in moderately injured myocytes can be reversed upon removal of the injurious agent and restoration of the cellular adenosine triphosphate level; and (3) changes in catecholamines mediated by an intact nervous system are not required for an increase in $\beta$-adrenergic receptor density in the setting of impaired energy metabolism. (Circ Res 57: 640–645, 1985)

INTEREST has developed in the pathobiology of adrenergic receptors during myocardial ischemia because of the possible role of altered adrenergic function in arrhythmogenesis and loss of myocardial cell viability following an ischemic insult (Opie et al., 1979; Muntz et al., 1984). Previous experimental studies have shown that coronary occlusion for 30 minutes to 1 hour is associated with the following alterations in ischemic myocardium: (1) release of catecholamines from nerve terminals (Muntz et al., 1984), and (2) an increase in the density of $\alpha$- or $\beta$-adrenergic receptors (BAR) in various species (Corr et al., 1981; Mukherjee et al., 1979, 1982). However, several questions remain to be answered regarding the mechanisms involved in the adrenergic alterations and the consequences of the alterations. We reasoned that these issues could be optimally addressed in a more controlled manner in an in vitro preparation, i.e., the cultured neonatal rat cardiac myocyte model subjected to metabolic inhibition. Specifically, we asked the questions: (1) what is the relationship between an increase in $\beta$-adrenergic receptors and the severity of metabolic inhibition, impaired energy metabolism, and cell injury? (2) can an increase in $\beta$-adrenergic receptor number be reversed? and (3) is an intact nervous system required for the $\beta$-adrenergic receptor change?

Methods

Cultured Myocyte Model

Neonatal rat myocardial cells were cultured by a modification of the method of Harary and Farley (1963). Hearts were isolated from 2- to 3-day-old rats, the atria were removed, and the ventricles were minced in a HEPES-buffered balanced salt solution. The myocardial cells were dispersed by incubation in 0.06% pancreatin (GIBCO) and 0.03% collagenase type II (Worthington) at 37°C for 20 minutes. The initial supernatant was removed and discarded. The mince was incubated with fresh pancreatin-collagenase for 20 minutes at 37°C. These steps were repeated a total of 4 times. The cell suspensions from each digestion were combined and centrifuged. The pellet was suspended in culture medium consisting of 68% Dulbecco's modified Eagle's medium with 18 mEq glucose (GIBCO), 0.03% collagenase type II (Worthington) at 37°C for 20 minutes. The initial supernatant was removed and discarded. The mince was incubated with fresh pancreatin-collagenase for 20 minutes at 37°C. These steps were repeated a total of 4 times. The cell suspensions from each digestion were combined and centrifuged. The pellet was suspended in culture medium consisting of 68% Dulbecco's modified Eagle's medium with 18 mEq glucose (GIBCO), 17% medium 199 with Earle's salts (GIBCO), 10% horse serum, 5% fetal calf serum, and antibiotics (penicillin 10,000 U/ml, streptomycin 10,000 µg/ml). The
cell suspension was plated for up to 3 hours (differential plating period), and the unattached cells (myocyte enriched) were removed and replated on 10-cm culture dishes (Blondel et al., 1971). The cells reached confluency at 72 hours. With this technique, the percent of beating myocytes in each preparation was approximately 80% or greater.

Experiments on 3-day-old cultures were initiated by replacing the growth medium with medium that contained either the mitochondrial respiratory inhibitor, potassium cyanide (KCN) (7.5 × 10^-4 M), and the nonmetabolized glucose derivative, 2-deoxyglucose (DOG) (4.5 × 10^-3 M), or the glycolytic enzyme inhibitor, iodoacetate (IAA) (3 × 10^-5 M) for up to 4 hours. The doses of metabolic inhibitors were chosen on the basis of studies documenting the progression of injury with these agents in this model (Chien et al., 1985). Control cultures from the same litters were maintained in growth medium without metabolic inhibitors. In other experiments, the medium containing metabolic inhibitors was replaced with fresh medium without metabolic inhibitors, and the cultures were maintained for another 24 hours. Experiments with control and treated cultures were performed concurrently.

β-Adrenergic Receptor Assays

Binding assays were performed in cell homogenates using modifications of the methods of Maguire et al. (1976). All culture dishes were rinsed twice with warm 5 mM HEPES—1 mM MgSO4, pH 7.4. The cells were scraped from the surface of the dishes with a rubber policeman in 5 ml buffer, and 5 ml more buffer was added. Samples were taken for cell counts and for protein determinations by the method of Lowry et al. (1951). The cells were centrifuged in a table-top centrifuge at 1000 g for 5 minutes. The pellet was resuspended and gently homogenized by hand in a Dounce apparatus (eight strokes). Cells harvested from two 10-cm culture plates were used for each assay.

Binding assays were performed in glass tubes. The ligand used was [125I]iodocyanopindolol ([125I]CYP). Preparations were synthesized and provided us by Dr. S.L. Petrovic (Department of Physiology, UTHSCD) (Petrovic et al., 1983) or were obtained from New England Nuclear. For determination of nonspecific binding, 10^-5 M alprenolol was used. The incubation mixture consisted of: 50 μl radiolabeled ligand, 50 μl buffer or alprenolol, 300 μl buffer (50 mM HEPES—1 mM MgSO4, pH 7.4), and 100 μl cell membranes in a total of 500 μl. Incubations were continued typically for 45 minutes at 37°C. The reaction was terminated by filtering the incubation mixture with Whatman GF/C glass microfiber filters. The filters were washed five times with 4 ml of 20 mM potassium phosphate—1 mM MgSO4, pH 7.4, at 37°C. Radioactivity was measured in a Beckman LS 7500 scintillation counter.

Binding curves and Scatchard plots were constructed for determination of maximum number of sites (Bmax) and affinity constant (Kd) of ligand binding (Williams and Lefkowitz, 1978). The half-life of 125I and the efficiency (65.8%) of the scintillation counter for 125I were taken into account in the calculations. Each assay was performed in duplicate. Bmax was converted from fmol/mg protein to sites/cell using the following factors: (1) 1 mg protein = 10^9 cells (determined experimentally), and (2) 1 mol = 6.022 × 10^23 sites (Avogadro’s number).

Adenosine Triphosphate (ATP) Assays

The medium was removed from the dishes and the myocardial cells were rinsed twice with 1 ml saline. The cells were scraped directly into 2 ml of ice cold 7% perchloric acid, and the extracts were obtained by centrifugation. The time required to terminate each sample was less than 25 seconds. The extract was neutralized with 5 N KOH, filtered with a Waters SepPak, and injected directly into a Waters high-pressure liquid chromatograph. Quantification and separation of the adenine nucleotides were accomplished with a Waters 10-μm Bondapak C-18 reverse phase column (Anderson and Murphy, 1976). The precipitate from the original extraction was solubilized in 1 ml of 0.1 N NaOH, and the protein concentration was measured by the method of Lowry et al. (1951). Each assay was performed in duplicate.

Statistical Analysis

Data from experimental groups and matched controls were compared with a two-tailed Student’s t-test. For two group comparisons, differences were considered significant when P < 0.05. For multiple group comparisons, the level of statistical significance was modified by the Bonferroni correction for multiple comparisons (Wallenstein et al., 1980). Experiments involving multiple group comparisons also were evaluated by a one-way analysis of variance. For correlation analysis between two measurement parameters, Pearson’s correlation coefficient was used. Data were expressed as the mean ± SE.

Results

Characterization of BAR Binding

Incubation of [125I]CYP with membrane-containing homogenates of control myocytes resulted in specific binding which was approximately 80% of total binding at saturation (Fig. 1). Specific binding reached equilibrium within 30–45 minutes. Calculation of the Kd from Scatchard plots and association-dissociation curves gave similar values. With the two preparations of [125I]CYP used in our studies, Kd for control cells was in the range of 160–180 pm and 20–60 pm, respectively. With both preparations, Bmax and time to equilibrium were similar. In competition experiments, the order of potency of agonists and antagonists was typical of a β-adrenergic receptor. The agonist-binding curves are shown in Figure 2. Using d- and l-epinephrine, stereospecificity was observed (Fig. 2). The average (n = 3 experiments) concentrations of agonists producing 50% inhibition of [125I]CYP binding (IC50) were: l-isoproterenol, 1 × 10^-7 M; l-norepinephrine, 7.9 × 10^-7 M; l-epinephrine, 1.4 × 10^-6 M; and d-epinephrine, 3.3 × 10^-5 M. Competition studies with antagonists yielded the following average IC50 values: l-propranolol, 2 × 10^-4 M (n = 3); d-propranolol, 5 × 10^-4 M (n = 1); and phentolamine, 2 × 10^-4 M (n = 3). In binding studies performed with homogenates of myocytes treated with DOG and KCN or with IAA, specific binding reached equilibrium within 30–45
Changes in BAR and ATP with Metabolic Inhibition

After incubation of cultured myocytes with KCN and DOG for 4 hours, there was a decrease or cessation of beating activity with preservation of cellular morphology when the cultures were viewed with the inverted polarized light microscope. The KCN- and DOG-treated cells exhibited a 41% decrease in ATP and increases of 43% in $B_{\text{max}}$ and 32% in $K_D$ (Table 1). In other experiments ($n = 8$), normal cells were scraped off the culture dishes, and KCN and DOG were added directly to the broken cells during the BAR assay. There was a significant increase in $K_D$ (214 ± 8 vs. 159 ± 7 pM), but no change in $B_{\text{max}}$ (13,018 ± 1329 vs. 13,181 ± 877 sites/cell) compared to controls. These data indicate that the change in $B_{\text{max}}$ is related to metabolic alterations induced by KCN and DOG in the intact cells, whereas the change in $K_D$ is related to a direct effect of these inhibitors on $[{}^{125}\text{I}]$CYP binding in the assay system.

In the IAA experiments, myocytes retained beating activity after incubation for 1.2 hours (70 minutes) with the inhibitor. Between 1.2 and 2 hours, beating activity decreased or ceased, many myocytes exhibited increased cytoplasmic granularity, and some myocytes showed more severe changes. At 3 hours, most myocytes exhibited severely distorted morphology, including rounded shape and surface blebs. ATP levels were moderately reduced after 1.2 hours and severely reduced at 1.5 to 2 hours and 3 hours (Table 2). The $B_{\text{max}}$ of BAR was unchanged after 1.2 hours, significantly increased after 1.5-2 hours, and significantly reduced after 3 hours of
IAA treatment compared to controls (Table 2). The $K_p$ with IAA-treated cells was unchanged compared to matched controls (Table 2). There were no differences in the protein yields between control cultures and those treated with KCN and DOG or with IAA. For example, protein contents (mg/60 mm plate) were: 0.37 ± 0.06 for control vs. 0.4 ± 0.06 for 4 hours KCN and DOG, and 0.35 ± 0.07 for control vs. 0.33 ± 0.05 for 1.5–2 hours IAA.

In the various experiments with KCN and DOG, and with IAA, there was no significant correlation between the $B_{\text{max}}$ of BAR and ATP concentration.

**Potential Reversibility of BAR and ATP Changes**

In another series of experiments, incubation with KCN and DOG for 4 hours again resulted in an increase in $B_{\text{max}}$ of BAR and a reduction in ATP (Table 3). In matched cultures, the medium containing KCN and DOG was removed after 4 hours, and the cells were maintained in medium without the inhibitors for an additional 24 hours. During the recovery period, the cultures resumed normal beating activity. Homogenates from these cells exhibited no differences in $B_{\text{max}}$ and $K_p$ of BAR or in ATP, compared to controls (Table 3). These data indicate that the changes in BAR and ATP can be reversed after removal of KCN and DOG.

**Discussion**

The general purpose of the present study was to examine potential relationships among BAR changes, ATP concentration, and stages of cell injury. Our data indicate that the density of BAR initially is increased in metabolically impaired myocytes, and subsequently is decreased in such myocytes after the onset of irreversible injury, and that the increased BAR density in moderately injured myocytes is potentially reversible, if the injurious stimulus is removed and ATP concentration is returned toward normal.

Treatment of cardiac myocytes for 4 hours with KCN (7.5 x 10^{-4} M) and DOG (4.5 x 10^{-2} M) resulted in a 43.5% (Table 1) to 45% (Table 3) increase in ATP number which was associated with minimal morphological alterations and moderate reduction in ATP. The changes in both BAR and ATP were reversed upon removal of the metabolic inhibitors. In a previous study, we observed that myocytes treated with similar doses of KCN and DOG for 4 hours did not release creatine kinase (CK), released only small amounts of [3H]arachidonate from membrane phospholipids, and were capable of resynthesizing ATP after 24 hours in medium free of KCN and DOG (Chien et al., 1985). Eight to 12 hours of treatment with these inhibitors were required to produce marked release of CK and arachidonate and persistent depression of ATP levels (Chien et al., 1985).

The course of injury was more rapid when myocytes were exposed to IAA (3 x 10^{-5} M) than to DOG and KCN. Previously, we observed that IAA exposure resulted in minimal release of arachidonate and CK after 1 hour, moderate release after 2 hours, and marked release after 4 hours (Chien et al., 1985). ATP was resynthesized to normal levels after 1 hour, whereas there was no resynthesis after 2 and 4 hours of exposure to IAA. Severe morphological damage developed in increasing numbers of myocytes over the 4-hour period. In the present study, IAA treatment resulted in a 33% increase in BAR numbers after 1.5–2 hours of exposure, and a subsequent decrease in BAR after 3 hours. Testing for potential reversibility of the BAR increase in IAA-treated myocytes was not possible because cultures treated with IAA for 1.5–2 hours or longer exhibited progressive cellular degeneration after removal of the IAA.

The relationship between changes in BAR and progression of injury in the cultured myocyte model suggests that BAR density increases during the reversible phase of injury, remains increased during a transitional stage of injury, and decreases below control levels after the myocytes become irreversibly injured. The degree of coupling of the increased BAR to metabolic activity in the cultured myocytes remains to be determined. However, the finding that increased BAR density develops relatively early in the course of injury in cultured myocytes is consistent with in vivo observations, i.e., that agonist stimulation of the increased numbers of BAR in ischemic myocardium results in increased metabolic activity in the form of increased formation of cyclic adenosine monophosphate and activated phosphorylase (Mukherjee, 1982).

**Table 3**

<table>
<thead>
<tr>
<th>Groups</th>
<th>$B_{\text{max}}$ (sites/ell)</th>
<th>$K_p$ (pm)</th>
<th>ATP (nm/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 8)</td>
<td>12,187 ± 603</td>
<td>27 ± 3</td>
<td>29 ± 3</td>
</tr>
<tr>
<td>KCN + DOG, 4 hr (n = 8)</td>
<td>17,687 ± 658</td>
<td>44 ± 2</td>
<td>16 ± 4</td>
</tr>
<tr>
<td></td>
<td>$P &lt; 0.001^*$</td>
<td>$P &lt; 0.001$</td>
<td>$P &lt; 0.025$</td>
</tr>
<tr>
<td>Control (n = 8)</td>
<td>13,025 ± 359</td>
<td>27 ± 3</td>
<td>29 ± 4</td>
</tr>
<tr>
<td>24 hr after KCN + DOG</td>
<td>13,175 ± 333</td>
<td>28 ± 3</td>
<td>27 ± 5</td>
</tr>
<tr>
<td>(n = 8)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>ANOVA</td>
<td>$P &lt; 0.001$</td>
<td>$P &lt; 0.001$</td>
<td>$P &lt; 0.10$</td>
</tr>
</tbody>
</table>

*Results of statistical analysis of each group of treated myocytes and concurrent controls using Student's $t$-test with level of significance at $P < 0.025$ according to the Bonferroni correction for two comparisons.

† Results of analysis of variance testing for the presence of significant difference(s) among all four groups.
It is possible that the increase in density of β- and α-adrenergic receptors in ischemic myocardium of various species is a phenomenon that results from upregulation secondary to loss of catecholamines following release from nerve terminals in ischemic myocardium. This possibility is not supported by the observations in the cultured myocyte model, since the increase in BAR following metabolic inhibition occurred in a model without an intact nervous system. We cannot exclude the possibility that catecholamines were present in the culture medium or that they may have exerted some effects in the culture system. However, differences in catecholamines between treated and control cultures would be unlikely in individual experiments in which the same culture medium was used for both groups.

Another possibility is that the increase in adrenergic receptor density is related to decreased availability of high-energy phosphates. Specifically, ATP deficiency may prevent internalization of membrane receptors, a process which is known to be ATP dependent (Chuang et al., 1980; Limas and Limas, 1984). In the present study, a general association was established between increased BAR density and ATP reduction. However, there was no statistically significant linear relationship between the magnitude of the BAR increase and the ATP decrease. Thus, it is not clear whether the ATP reduction is simply a manifestation of cell injury, or whether some more specific link exists between changes in certain pools of ATP and alterations in adrenergic receptors.

A third possibility is that the increases in β- and α-adrenergic receptor density in ischemic myocardium develop as a result of changes in membrane fluidity in reversibly injured myocytes, and that such membrane changes "unmask" adrenergic receptors (Strittmatter et al., 1979) or otherwise alter the interaction of ligands and the receptors, such that increased numbers of adrenergic receptors are available for ligand binding. Thus, the present study has established that BAR density in myocytes increases with ATP reduction produced by two different types of metabolic inhibition, although there is a poor correlation between absolute changes in BAR number and ATP concentration. Furthermore, we showed that the increase in BAR density develops in reversibly injured myocytes and can be reversed upon removal of metabolic inhibition. The increase in BAR density does not necessarily occur in response to loss of catecholamines. These findings are compatible with the possibility that the increase in BAR density in ischemic myocardium is capable of mediating metabolic and electrophysiological activity that contributes to the progression of myocardial infarction and the development of arrhythmias, and that these abnormalities are potentially reversible if the ischemia is relieved. However, further work is needed to establish the mechanism of the BAR increase and to define more precisely the metabolic and electrophysiological consequences of this alteration. Nevertheless, the present study has established a useful model and has documented the dynamic nature of changes in BAR density during the evolution of myocyte injury.

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Buja et al. /β-Adrenergic Receptor Changes in Injured Myocytes

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