Forum Original Research Communication

Role of Adenosine Receptor Activation in Antioxidant Enzyme Regulation During Ischemia–Reperfusion in the Isolated Rat Heart

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ABSTRACT

The aim of the present study was to investigate the protective role of pharmacological preconditioning on antioxidant enzymes using A1 and A3 adenosine receptor agonists in the recovery of the isolated myocardium after cardioplegic ischemia. Two different modes of preconditioning were studied: isolated rat hearts were perfused with A1 receptor agonist 2-chloro-N6-cyclopentyladenosine (CCPA) or A3 2-chloro-N6-(3-iodobenzyl) adenosine-5’-N-methyluronamide (Cl-IB-MECA) (1 nM), followed by cardioplegic ischemia and reperfusion (30 min each) (perfusion mode), or CCPA or Cl-IB-MECA (100 µg/kg) were injected intravenously 24 h before the experiment (injection mode). Hearts treated with CCPA improved in terms of mechanical function, infarct size, ATP levels, superoxide dismutase, and catalase (p < 0.005) in both modes of administration. Cl-IB-MECA was beneficial mainly in the injected group. Reduced damage to the mitochondria in the CCPA-treated hearts was observed using electron microscopy evaluation. In the Cl-IB-MECA-injected hearts, mitochondrial damage was moderate. CCPA in both modes of treatment and Cl-IB-MECA in the injected mode were beneficial in protecting the perfused isolated rat heart, subjected to normothermic cardioplegic ischemia. This protection was partially related to the higher myocardial activity of superoxide dismutase and catalase. Antioxid. Redox Signal. 6, 335–344.

INTRODUCTION

STRATEGIES FOR PROTECTING THE HEART from surgically related ischemia and reperfusion injury have evolved over the past decade. However, patients with severe cardiac injury require prolonged operative–ischemic time. Hence, specific therapy to adequately restore contractile function and morphology is needed.

Reperfusion of ischemic myocardium may induce a marked increase in the generation of reactive oxygen species (ROS) in both experimental and clinical heart failure (11, 18). ROS play an important role in the progression and aggravation of heart failure, causing the oxidation of membrane phospholipids, proteins, and DNA. ROS can also induce contractile dysfunction and myocardial structural damage (1). Cardiomyocyte injury is caused by highly reactive hydroxyl radical (·OH), generated from superoxide anion (O2·−) and hydrogen peroxide (H2O2) via the iron-mediated Fenton reaction together with calcium overload (14). The antioxidant capacity of myocytes undergoing heart failure, including the activity of myocardial antioxidant enzymes, is responsible for the extent of their susceptibility to oxidative stress. Superoxide dismutase (SOD) catalyzes the dismutation of O2·− to H2O2. Subsequently, H2O2 is reduced to H2O and O2 by a peroxidase such as glutathione peroxidase (GSH-Px), or catalase (8, 31, 32). Therefore, increasing the activity of intracellular anti-oxidants & REDOX SIGNALING Volume 6, Number 2, 2004 © Mary Ann Liebert, Inc.
dant enzymes seems a promising approach to achieve cardio-
protection.

Brief periods of myocardial ischemia induce a biphasic pattern of protection against prolonged ischemic injury, known as ischemic preconditioning (24). Adenosine, released endoge-
nously during brief ischemia from cardiomyocytes acting on
adenosine receptors, is recognized as one of the mediators of
preconditioning (9). It has been suggested that adenosine med-
iates cytoprotection by increasing the activity of antioxidant
enzymes (21). This idea became of interest when activation of
adenosine A₁ receptor-induced mitochondrial manganese SOD
(Mn SOD) elevation was thought to be a potential end effec-
tor of delayed preconditioning in rats (9, 22, 33). Selective
activation of adenosine A₁ and A₃ receptors protected the heart
against infarction (4, 19, 34, 36). Cardioplegia is used to pro-
tect the myocardium against ischemic injury during cardiac
surgery (10). In the isolated rat heart, it was reported that either
preconditioning or cardioplegia alone protected posts ischemic
contractile and vascular function to the same extent, but their
combination showed no additional protection (17). In con-
trast, preconditioning followed by cardioplegic ischemia of
human myocardium appeared beneficial in terms of myocar-
dial ATP content, in either coronary bypass or valve operations
(20, 39). It is therefore of interest to carry out a comparative
analysis of the role of antioxidant enzymes in pharmaco-
logical preconditioning, induced by activation of the adenosine
A₁ and A₃ receptor subtypes. The specific goal of this study was
to define conditions under which adenosine A₁ receptor agonist, 2-chloro-N^6-cyclopentyladenosine (CCPA), or the A₃
receptor agonist, 2-chloro-N^6-(3-iodobenzyl)adenosine-5'-N-
methylyuronamide (Cl-IB-MECA), reduce cardioplegic ischemic
damage in the isolated rat heart. Drugs were administered using
two different modes: perfusion immediately before ischemia
or intravenous injection 24 h before the ischemic insult. Our
results support previous findings that adenosine receptor ac-
tivation, particularly with CCPA, reduces cardioplegic post-
ischemic ventricular dysfunction, suggesting that it improves
the antioxidant reserves of the heart.

MATERIALS AND METHODS

Animal procedures

Animal care complied with the Principles of Laboratory
Animal Care and the Guide for the Care and Use of Laborato-
ry Animals.

Adult male rats (200–250 g) of the Wistar strain were used
in this study. Heparin (100 U/rat) was administered intra-
peritoneally. After 30 min, the animals were anesthetized with
diethyl ether. Their hearts were rapidly excised and mounted on
the stainless steel cannula according to the modified Langen-
dorff system. Retrograde aortic perfusion was initiated at a
perfusion pressure of 90 cm H₂O with modified Krebs–
Henseleit buffer solution (KH), which contained 118 mM NaCl,
25 mM NaHCO₃, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄ • 7H₂O,
2.5 mM CaCl₂, 4.7 mM KCl, 11.1 mM glucose, 0.5 mM
Na₂EDTA bubbled with a mixture of O₂/CO₂ (95%:5%), re-
sulting in a pH of 7.35–7.40. P₀ and Pco₂ values in the per-
fusion medium were 550–650 and 25–30 mm Hg, respectively
(26). The pulmonary artery was incised to facilitate drainage.
The cardioplegic solution was KH solution with 16 mM KCl
and 106.7 mM NaCl to maintain constant osmolarity. Tempe-
ration was maintained at 37 ± 0.5°C, by placing a thermostatic
water jacket around the perfusate reservoir and the isolated
heart. Heart temperature was monitored with a micro thermo-
couple in the right ventricle connected to a digital thermom-
eter (Webster Laboratories, Altadena, CA, U.S.A.).

 Epicardial pacing wires were connected to the right ventri-
cle and the aortic cannula. After completion of the preparation
at 300 bpm (5 V, 10-ms duration), using an external Harvard
stimulator (Edenbridge, Kent, U.K.), pacing commenced. A
latex balloon filled with water was inserted into the left ven-
tricular cavity through a small incision in the left atrium and
connected to a Statham Medical P132284 pressure transducer
(Mennen Medical, Inc., Clarence, NY, U.S.A.). The balloon was
tied and inflated to a volume producing a diastolic pressure
of 0–5 mm Hg. Left ventricular developed pressure (LVDeVP)
was continuously monitored during the experiment and recorded
every 10 min using AT-CODAS Software (Dataq Instruments,
Inc., Akron, OH, U.S.A.). Coronary flow (CF) was measured by
collecting the effluent into a calibrated beaker for 1 min at 30
min of the stabilization period and at 1, 10, 20, and 30 min of
reperfusion (12). The biochemical parameters tested were cre-
tine kinase (CK) immediately before ischemia and at 1 and
30 min of reperfusion, using commercial kits.

We conducted a dose-finding pilot study before the main
experiments, in which we tested different concentrations of
the studied compounds and chose those with the best results
in terms of LVDeVP, CF, and arrhythmias.

The experimental compounds were CCPA, Cl-IB-MECA, 1
mM in KH (perfused) or 100 µg/kg injected intravenously. The
A₁ and A₃ receptor antagonists 8-cyclopentyl-1,3-dipropyl-
xanthine (DPCPX) and 2,3-diethyl-4,5-dipropyl-3-thiocarboxyl-
ate-6-phenylpyridine-5-carboxylate (MRS1523) were tested
either alone or in combination with CCPA and Cl-IB-MECA.

Experimental protocol

Perfused groups. Isolated hearts were perfused with
KH solution for 30 min (stabilization period) with and without
the drugs. The cardioplegic solution was administered for 2
min, and no flow global ischemia at 37°C was maintained for
30 min. At the end of the ischemic period, hearts were reper-
fused with KH for 30 min.

Three groups of hearts were studied (n = 10 in each group):
(a) control hearts undergoing ischemia/reperfusion (I/R); (b)
hearts perfused with CCPA; and (c) hearts perfused with Cl-
IB-MECA.

Injected groups. Rats were injected intravenously with
the experimental drug 24 h before the experiment. Isolated hearts
were perfused as described above. At the end of each experi-
ment, myocardial tissue was frozen in liquid nitrogen and kept at
−70°C until analyzed. Tissue specimens were also conserved in
formaldehyde and glutaraldehyde for microscopic analysis.

Animals in these groups were injected with the following
drugs: (a) CCPA (n = 10); (b) DPCPX (n = 3); (c) CCPA +
DPCPX (n = 6); (d) Cl-IB-MECA (n = 10); (e) MRS1523
(n = 3); and (f) Cl-IB-MECA + MRS1523 (n = 6).
ATP concentration

Myocardial tissue was harvested in 1 ml of cold 5% trichloroacetic acid. The cell extract was used to measure ATP content by using a luciferin-luciferase bioluminescence kit (ATP Bioluminescence Assay Kit CLSH, Boehringer Mannheim).

Measurement of antioxidant enzymes

To examine whether antioxidative enzymes participate in the pharmacological ischemic preconditioning, we measured the activities of three antioxidant enzymes: SOD, catalase, and GSH-Px. Heart biopsy (0.1 g) was placed in a measured volume of ice-cold Tris-sucrose buffer containing 250 mM sucrose, 10 mM Tris-HCl, 1 mM EDTA, 0.1 mM dithiothreitol, and 0.1 mM phenyl methyl sulfoxide, at pH 7.5. The tissue was homogenized and centrifuged at 3,000 g for 15 min at 4°C. The decanted supernatant was then centrifuged at 10,000 g for 20 min at 4°C, and at 105,000 g for 1 h at 4°C in a Beckman L-80 ultracentrifuge. The obtained supernatant was used to assay SOD, catalase, and GSH-Px. SOD activity was measured in an assay medium containing 14.4 µM cytochrome c, 71.4 µM xanthine, 35.7 mM buffer phosphate (pH 7.8), and 1–5 µg of enzyme protein (total volume 225 µl). The reaction was initiated with the addition of 3 mU of xanthine oxidase, and changes in absorption of reduced cytochrome c were monitored spectrophotometrically (550 nm). Catalase was estimated by measuring the decrease in H₂O₂ absorption at 240 nm. The final concentration of the assay medium (total volume 200 µl) was 50 µmol/L potassium phosphate buffer (pH 7.0) and 30–50 µg of enzyme protein. The reaction was started by adding 200 µl of 0.068% H₂O₂. GSH-Px was determined by estimating the decrease in NADPH absorption at 340 nm. Final concentrations of the components used in the assay medium for GSH-Px (total volume 200 µl) were 60 mM phosphate buffer pH 7, 0.5 mM EDTA, 0.25 U/ml glutathione reductase, 0.1 mM glutathione, 0.15 mM NADPH, 0.01% NaHCO₃, and 10–50 µg of protein enzyme. The reaction was initiated by adding 20 µl of 12 mM tert-butyl hydroperoxide and the decrease in absorption followed for 3 min at 340 nm (7).

Measurement of irreversible ischemic injury

Upon termination of the experiment, the heart was infused (2 ml) into the coronary vasculature through the side arm of the aortic cannula with a 1% solution of 2,3,5-triphenyltetrazolium chloride (TTC) in phosphate buffer at 37°C and cut into sections (width 0.8 mm). Slices were placed in an identical TTC solution at 37°C for 30 min. TTC stained the viable tissue red, whereas the necrotic tissue remained discolorated. Sections were fixed overnight in 2% paraformaldehyde. Sections were then digitally photographed using a Fugi Finepixs1pro camera, at a resolution of 1,400 × 960 pixels and quantified with IMAGE J 5.1 software. The area of irreversible injury (TTC-negative) is presented as a percentage of the entire area of the section (23).

Electron microscopy

At the end of procedures, hearts were immediately perfusion-fixed with 2.5% glutaraldehyde in KH solution for 5 min. Left ventricular tissue was dissected out, and sections were further fixed for 2 h with 2.5% glutaraldehyde in 0.1 M sodium cacodylate for 1 h, postfixed in 1% osmium tetroxide in the same buffer for 1 h, and stained in 0.5% uranyl acetate. Cells were dehydrated in a series of ascending alcohol concentrations infiltrated with Epon-Araldite epoxy resin, and heat-polymerized. Sections were cut using an ultramicrotome, poststained with uranyl acetate and lead citrate, and then examined (30). The volume density of cellular components was determined by the point-count method (38). In brief, a transparent grid of test points was laid over each micrograph. The number of test points falling on an individual structure was recorded, as was the total number of test points available on the test grid. The volume occupied by each component was equivalent to the number of points falling on that structure divided by the total number of test points available on the test grid. This value was expressed as a percentage. Three types of mitochondria were analyzed: mature mitochondria, containing well-developed cristae; vacuolated mitochondria; and irreversibly damaged mitochondria with electron-dense deposits containing calcium. Two types of myofibrils, myofibrils with regular appearance and myofibrils with disruption, were also determined.

Statistical analysis

All results are expressed as means ± SEM. Values of the stabilization period are considered as 100%. ANOVA was used to compare groups; the Bonferroni test was used to compare differences between the groups at every checked point.

RESULTS

Table 1 summarizes LVEDP and CF baseline absolute values in the different groups before the induction of ischemia and reperfusion at 30 min after perfusion. No significant differences were found in hemodynamic performance at baseline between control I/R and hearts that were injected with CCPA or CI-IB-MECA with and without their specific antag-
onist (DPCPX or MRS1523) 24 h before the experiment. However, hearts that were perfused with CCPA or CI-IB-MECA showed elevated LVDevP and CF ($p < 0.05$ versus control).

### Mechanical recovery

**Drug-perfused groups.** LVDevP and CF in absolute values were higher in the drug-perfused hearts (Figs. 2 and 3). Following cardioplegic ischemia, statistically significant differences between percent recoveries of LVDevP of the CCPA-perfused hearts compared with the CI-IB-MECA or control hearts were found (Fig. 2). At 30 min of reperfusion, LVDevP was $78 \pm 3\%$ and $55 \pm 9\%$ in CCPA- and CI-IB-MECA-perfused hearts, respectively, versus $59 \pm 5\%$ in controls ($p < 0.04$). CF recovery of the drug-perfused hearts was higher than the control reperfusion (Fig. 3).

**Drug-injected groups.** Recovery of the hearts treated with CI-IB-MECA or CCPA 24 h before cardioplegic ischemia was better in terms of LVDevP and at 30 min following ischemia and reperfusion it was $80 \pm 3.3\%$ and $75 \pm 4.6\%$ in CCPA- and CI-IB-MECA-injected hearts, respectively, versus $59 \pm 5\%$ in controls ($p < 0.005$) (Fig. 2). No difference was found in end diastolic pressure between all the groups in the stabilization or in the reperfusion period, except for the CCPA-injected hearts at 10 min of reperfusion, which had lower lower end diastolic pressure compared with controls ($13.2 \pm 3.5$ versus $23 \pm 6$ mm Hg, $p < 0.05$).

CF also improved ($91 \pm 21\%$ and $78 \pm 7\%$ in CCPA- and CI-IB-MECA-injected hearts versus $64 \pm 4\%$ in controls, $p < 0.004$) at 30 min of recovery (Fig. 3). DPCPX and MRS1523 alone did not exert any influence at any point compared with controls. The addition of DPCPX to CCPA or MRS1523 to CI-IB-MECA abolished all the favorable changes mentioned above, and the results were similar to those of controls (data not shown).

### Biochemical markers of ischemia and reperfusion damage

CK activity (U/min) in effluent increased in all groups after I/R. CK release to the coronary effluent at 10 min of reperfusion was 6.4 and 2.74 times higher in control I/R hearts than CCPA- or CI-IB-MECA-injected hearts (Fig. 4). Drug perfusion was not as effective as the injection mode in ameliorating enzyme release in the coronary effluent (Fig. 4).

#### Table 1. Preischemic Values

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>LVDevP (mm Hg)</th>
<th>CF (ml/min)</th>
</tr>
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<tbody>
<tr>
<td>Control I/R</td>
<td>10</td>
<td>104 ± 9.7</td>
<td>9.9 ± 0.8</td>
</tr>
<tr>
<td>CCPA perfused</td>
<td>10</td>
<td>130 ± 8*</td>
<td>20.3 ± 2.0*</td>
</tr>
<tr>
<td>CI-IB-MECA perfused</td>
<td>10</td>
<td>131 ± 8*</td>
<td>17.3 ± 1.6*</td>
</tr>
<tr>
<td>CCPA injected</td>
<td>10</td>
<td>101 ± 7.4</td>
<td>9.3 ± 0.7</td>
</tr>
<tr>
<td>CI-IB-MECA injected</td>
<td>10</td>
<td>119 ± 12.6</td>
<td>12.0 ± 1.74</td>
</tr>
<tr>
<td>CCPA + DPCPX injected</td>
<td>6</td>
<td>100 ± 14.0</td>
<td>9.5 ± 0.65</td>
</tr>
<tr>
<td>CI-IB-MECA + MRS1523 injected</td>
<td>6</td>
<td>108 ± 4.3</td>
<td>9.7 ± 0.86</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE. *$p < 0.05$ versus control I/R.

#### FIG. 2. LVDevP expressed as % of preischemic value in both modes of treatment with adenosine receptor agonists (perfused or injected). LVDevP at various time points following cardioplegic ischemia is presented. Values represent means ± SE. #*$p < 0.05$ versus stabilization from the same group.
Antioxidant defense enzymes in the myocardium

Ischemia and reperfusion induced the elevation of antioxidant enzymes. Injected hearts treated with CCPA induced marked activity of SOD and catalase, but not GSH-Px at baseline (Fig. 5). CCPA was the most effective treatment. The perfusion of CCPA or CI-IB-MECA induced elevated activity of GSH-Px at baseline, but after ischemia this activity was similar to the controls.

Cellular ATP content

ATP levels fell significantly after ischemia and reperfusion. Pretreatment with CCPA or CI-IB-MECA either in the perfusate or in the injection mode prevented a decrease in ATP levels (Fig. 6).

Irreversible ischemic injury

TTC staining revealed that the use of adenosine receptor agonists lowered the amount of irreversible ischemic injury in all treated groups. Drug perfusion or injection was accompanied by a reduction in regions of irreversible ischemic injury compared with control I/R hearts. As shown in Fig. 7, CCPA injection proved to be the most effective treatment (11.7 ± 2.6% versus control 1.7 ± 0.3%, \( p = 0.001 \)). Typical sections showing viable and necrotic areas are shown (Fig. 7).

Morphological markers of ischemia and reperfusion damage

Using electron microscopy, we found that ischemia and 30 min of reperfusion caused markedly higher damage in con-
controls than in the other groups. Mitochondria of many I/R treated hearts (Fig. 8A) were distorted with the swelling of many I/R treated hearts and contained electron-dense inclusions (calcium phosphate electron-dense deposits). Injection of hearts 24 h before the ischemic insult with A1 receptor agonist CCPA significantly decreased mitochondrial damage. Well developed cristae were

FIG. 5. Enzymatic activity of SOD (a), catalase (b), and GSH-Px (c) in hearts subjected to 30 min of ischemia followed by 30 min of reperfusion after treatments with adenosine receptor agonists. Antioxidant enzymatic activity was determined in myocardial extracts after 30 min of perfusion (stabilization), after 30 min of ischemia and 1 min or 30 min of reperfusion. Values represent means ± SE (n = 5 in each group). * p < 0.05 versus control I/R at the same time. # p < 0.05 versus stabilization from the same group.

FIG. 6. ATP levels in hearts subjected to 30 min of ischemia followed by 30 min of reperfusion after treatments with adenosine receptor agonists. ATP was determined in myocardial extracts after ischemia and reperfusion. Values represent means ± SE (n = 5 in each group). # p < 0.05 versus stabilization from the same group. * p < 0.05 versus control I/R at the same time.

FIG. 7. Effect of I/R on myocardial infarction. Irreversible ischemia was determined by scanning the images of heart ventricular sections stained with TTC. Two representative images of the control I/R and CCPA-injected groups, revealing various degrees of myocardial ischemia, are presented. Hearts were subjected to 30 min of ischemia followed by 30 min of reperfusion. A significant area of damaged tissue is noticeable in the I/R myocardium of the control I/R compared with CCPA-injected heart (p < 0.005). The percentage of irreversible injury from the total area of the section is presented in the graph. Values represent percentage of irreversible injury in means ± SE (n = 10 hearts in each group). * p < 0.05 versus control I/R.
seen in many organelles, and calcium deposits were less evident in the matrix of the majority of mitochondria (Fig. 8B). Protection by adenosine A<sub>1</sub> receptor agonist Cl-IB-MECA was less pronounced (Fig. 8C). Table 2 describes a higher damage to the mitochondria and myofibrils in the I/R ischemic group compared with CCPA- or Cl-IB-MECA-injected hearts.

**DISCUSSION**

Diseases due to ischemia and reoxygenation (e.g., myocardial infarction and stroke) are a common cause of morbidity and mortality. Understanding the role of ROS in reoxygenation injury may lead to potential therapies improving public health (18). The present study provides evidence of the role of adenosine receptor activation in antioxidant enzyme regulation during cardioprotective I/R in the isolated rat heart, adapting a clinical protocol widely used for open-heart surgery.

Animal studies have provided direct evidence of the role of free radicals in the development of myocardial injury (1, 27). Cellular antioxidant defense includes enzymes such as SOD, catalase, and GSH-Px, as well as the nonenzymatic antioxidants tocopherols, carotenoids, retinol, melatonin, ubiquinol, ascorbic acid, and glutathione (15). Studies have reported a reduction of infarct size with antioxidants (2). Exogenous administration of SOD and catalase to both cardioprotective and perfusion solutions was found to be beneficial to the hearts (2, 13). Conflicting findings were obtained when SOD was added to the perfusion solution, alone or in conjunction with catalase, after myocardial ischemia. A major problem of these studies has been that the exogenous antioxidant enzymes cannot permeate cell sites where free radicals are generated (13). An alternative approach may be activation of endogenous antioxidant enzymes through a signal transduction mechanism. For example, Dana et al. found that the CCPA injection 24 h before ischemia induced a significant increase in myocardial MnSOD content, accompanied by reduced infarction (5). The major finding of the present study was that pretreatment with a single intravenous injection of A<sub>1</sub> receptor agonist (delayed protection) afforded better myocardial protection than the A<sub>3</sub>

### Table 2. Morphometric Analysis of Protective Effects of Adenosine A<sub>1</sub> and A<sub>3</sub> Receptor Activation in Myocardial Cells After I/R Damage

<table>
<thead>
<tr>
<th></th>
<th>Mm</th>
<th>Mv</th>
<th>MCa</th>
<th>MF&lt;sub&gt;r&lt;/sub&gt;</th>
<th>MF&lt;sub&gt;d&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control, no ischemia</td>
<td>28.2 ± 3.7</td>
<td>2.3 ± 2.8</td>
<td>—</td>
<td>56.3 ± 3.5</td>
<td>—</td>
</tr>
<tr>
<td>Control I/R</td>
<td>3.1 ± 4.6</td>
<td>15.3 ± 3.1</td>
<td>14.6 ± 2.5</td>
<td>12.1 ± 4.7</td>
<td>46.2 ± 5.6</td>
</tr>
<tr>
<td>CCPA injected</td>
<td>18.3 ± 2.8*</td>
<td>12.6 ± 2.2</td>
<td>4.5 ± 2.1*</td>
<td>38.1 ± 3.6*</td>
<td>17.3 ± 4.6*</td>
</tr>
<tr>
<td>Cl-IB-MECA injected</td>
<td>10.2 ± 4.3*</td>
<td>14.1 ± 1.8</td>
<td>8.4 ± 3.3*</td>
<td>28.6 ± 3.4*</td>
<td>27.1 ± 2.4*</td>
</tr>
</tbody>
</table>

Protective effects of adenosine A<sub>1</sub> and A<sub>3</sub> receptor activation were studied by the morphometric analysis of cardiac cells before (control, no ischemia) and after cardioprotective I/R. The analysis included the percent changes in mitochondrial morphology [mature mitochondria, containing well developed cristae (Mm); vacuolated mitochondria (Mv); irreversibly damaged mitochondria, containing calcium phosphate electron-dense deposits (MCa)] and percent changes in myofibrillar morphology [myofibrils with regular appearance (MF<sub>r</sub>) and myofibrils with disruption (MF<sub>d</sub>)]. Values are expressed as means ± SE.

*<i>p < 0.05 versus control I/R.</i>
adenosine receptor agonist or their perfusion before ischemia (immediate protection). This protection was confirmed by the reduction of damage to the heart in terms of left ventricular function, end diastolic pressure, CF; enzyme leakage, ATP levels, infarct size, microscopy findings, and antioxidant enzymes. We assume that the reduced tissue damage prevented the degradation of ATP, but measurements of ATP contents do not allow us to draw conclusions on the rates of ATP production and hydrolysis, and therefore this question remains open. This study provides an insight into the cellular mechanism responsible for conferring increased myocardial tolerance to lethal ischemic injury 24 h after transient activation of A1 receptor. This treatment induced the elevation of total SOD and catalase activity before oxidative stress, offering greater tolerance against ischemic and reperfusion injury. CCPA injection 24 h prior to ischemia induced a dramatic elevation of SOD, catalase, and GSH-Px also after I/R. These data indicate a role for the antioxidant enzymes in mediating the delayed cardioprotection observed 24 h after A1 receptor activation. Immediate A1 and A3 receptor activation elevated GSH-Px before ischemia, whereas delayed A3 receptor activation induced this effect after ischemia. The direct mechanisms of the intracellular signaling causing these various effects are not known, but elevation of various antioxidants implies that their protection mode of action is different.

The fact that ROS contribute to I/R injury suggests that increasing the content or activity of endogenous cellular antioxidant enzymes or lowering the amount of ROS should protect tissues from the deleterious effects of I/R injury. It has previously been shown that CCPA-induced enhancement of MnSOD activity at 24 h is mediated via a protein kinase C- and tyrosine kinase-dependent pathway in rabbits (6). Overexpression of MnSOD alone rendered the heart more resistant to I/R without affecting the activity of the other antioxidant enzymes (3). Adenosine A1 receptor activation reduced ROS and attenuated stunning in ventricular myocytes, as well as canine myocardium, following I/R (25, 28). In our work, I/R increased the activity of all the antioxidant enzymes, but only CCPA pretreatment 24 h prior to ischemia increased baseline SOD and catalase activity significantly compared with the other groups. These enzymes are one of the mechanisms responsible for the cardioprotection achieved by CCPA injection. The fact that GSH-Px was not markedly increased compared with that in control ischemic hearts indicates that the amount of these enzymes in the CCPA-injected hearts was sufficient to lower ROS-induced cardiotoxicity. A1 adenosine receptor activation (perfusion mode) elevated SOD, but not catalase, activity. GSH-Px increased in all groups after 30 min of I/R. It appears that the signaling pathways of cardiomyocyte protection are different after activation of A1 and A3 adenosine receptor subtypes.

Short-term pretreatment with adenosine has been shown to reduce infarct size, improve postischemic contractile function, and preserve metabolic or energy status of ischemic-reperfused myocardium (16, 37). These cardioprotective effects may be mediated by the activation of A1 and A3 receptors through the opening of ATP-sensitive potassium (KATP) channels, and consequent reduction of calcium overload (29, 36). A3 receptor stimulation with Cl-IB-MECA, a highly specific adenosine A3 receptor agonist, attenuated postischemic contractile dysfunction and CK release in buffered perfused rat hearts (35). In our work, many markers of cardiac damage were attenuated by adenosine receptor stimulation. ATP depletion was effectively prevented, and the amount of mitochondrial damage as seen by electron microscopy was reduced. The most efficacious treatment, as seen by our results for cardiac LVDevP, ATP, antioxidant enzymes, and TTC staining, was CCPA injection 24 h prior to I/R. In addition, necrotic cell death was reduced 10-fold in the CCPA-injected hearts. The myocardial morphology of these hearts, particularly the mitochondrial structure, was better preserved in CCPA-treated hearts, as is evident from the amount of intramitochondrial electron-dense deposits and vacuoles.

In summary, we have shown that transient activation of adenosine A1 and A3 receptors with the highly selective adenosine agonists CCPA and Cl-IB-MECA resulted in the development of an ischemia-tolerant state in the rat myocardium. CCPA injection 24 h prior to ischemia proved to be the most effective of all the experiments in terms of antioxidant enzyme activity, mechanical activity, and amelioration of irreversible ischemic damage. The potential therapeutic application of such a potent and sustained effect in patients at risk of myocardial ischemic damage during cardiac surgery is very promising and justifies further exploration.

ABBREVIATIONS

CCPA, 2-chloro-N6-cyclopentyladenosine; CF, coronary flow rate; CK, creatine kinase; CI-IB-MECA, 2-chloro-N6-(3-iodobenzyl)adenosine-5'-N-methyluronamide; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; GSH-Px, glutathione peroxidase; H2O2, hydrogen peroxide; I/R, ischemia/reperfusion; KH, Krebs–Henseleit buffer solution; LVDevP, left ventricular developed pressure; MnSOD, manganese superoxide dismutase; MRS1523, 2,3-diethyl-4,5-dipropyl-6-phenylpyridine-3-thiocarboxylate-5-carboxylate; 1/2O2, superoxide anion; ROS, reactive oxygen species; SOD, superoxide dismutase; TTC, 2,3,5-triphenyltetrazolium chloride.

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