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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 A_1 and A_3 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 A_1 receptor agonist 2-chloro-*N*^{6}-cyclopentyladenosine (CCPA) or A_3 2-chloro-*N*^{6}-(3-iodobenzyl) adenosine-5′-*N*-methyluronamide (Cl-IB-MECA) (1 n*M*), 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 ($^{\bullet}O_2^-$) and hydrogen peroxide (H_2O_2) 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 $^{\bullet}O_2^-$ to H_2O_2. Subsequently, H_2O_2 is reduced to H_2O and O_2 by a peroxidase such as glutathione peroxidase (GSH-Px), or catalase (8, 31, 32). Therefore, increasing the activity of intracellular antioxi-

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dant enzymes seems a promising approach to achieve cardioprotection.

Brief periods of myocardial ischemia induce a biphasic pattern of protection against prolonged ischemic injury, known as ischemic preconditioning (24). Adenosine, released endogenously 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 mediates cytoprotection by increasing the activity of antioxidant enzymes (21). This idea became of interest when activation of adenosine A_1 receptor-induced mitochondrial manganese SOD (Mn SOD) elevation was thought to be a potential end effector of delayed preconditioning in rats (9, 22, 33). Selective activation of adenosine A_1 and A_3 receptors protected the heart against infarction (4, 19, 34, 36). Cardioplegia is used to protect the myocardium against ischemic injury during cardiac surgery (10). In the isolated rat heart, it was reported that either preconditioning or cardioplegia alone protected postischemic contractile and vascular function to the same extent, but their combination showed no additional protection (17). In contrast, preconditioning followed by cardioplegic ischemia of human myocardium appeared beneficial in terms of myocardial 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 pharmacological preconditioning, induced by activation of the adenosine A_1 and A_3 receptor subtypes. The specific goal of this study was to define conditions under which adenosine A_1 receptor agonist, 2-chloro-N^6-cyclopentyladenosine (CCPA), or the A_3 receptor agonist, 2-chloro-N^6-(3-iodobenzyl)adenosine-5′-*N*-methyluronamide (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 activation, particularly with CCPA, reduces cardioplegic postischemic 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 Laboratory Animals.

Adult male rats (200–250 g) of the Wistar strain were used in this study. Heparin (100 U/rat) was administered intraperitoneally. 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 Langendorff system. Retrograde aortic perfusion was initiated at a perfusion pressure of 90 cm H_2O with modified Krebs–Henseleit buffer solution (KH), which contained 118 m*M* NaCl, 25 m*M* $NaHCO_3$, 1.2 m*M* KH_2PO_4, 1.2 m*M* $MgSO_4 \cdot 7H_2O$, 2.5 m*M* $CaCl_2$, 4.7 m*M* KCl, 11.1 m*M* glucose, 0.5 m*M* Na_2EDTA bubbled with a mixture of O_2/CO_2 (95%:5%), resulting in a pH of 7.35–7.40. P_{O_2} and P_{CO_2} values in the perfusion 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 m*M* KCl and 106.7 m*M* NaCl to maintain constant osmolarity. Temperature 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 thermocouple in the right ventricle connected to a digital thermometer (Webster Laboratories, Altadena, CA, U.S.A.).

Epicardial pacing wires were connected to the right ventricle 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 ventricular 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 creatine 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 n*M* in KH (perfused) or 100 μg/kg injected intravenously. The A_1 and A_3 receptor antagonists 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) and 2,3-diethyl-4,5-dipropyl-3-thiocarboxylate-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 reperfused 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 experiment, myocardial tissue was frozen in liquid nitrogen and kept at −70°C until analyzed. Tissue specimens were also conserved in formaldehyde and glutharaldehyde 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).

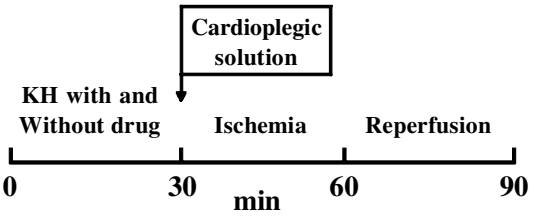

FIG. 1. Schematic representation of experimental protocol. On day 1, animals in the injected groups were pharmacologically preconditioned with an intravenous bolus injection of CCPA or Cl-IB-MECA with and without their specific antagonists DPCPX or MRS1523. On day 2, all animals were anesthetized and their isolated hearts were perfused with KH solution for 30 min (stabilization period) with and without the drugs. Cardioplegic solution was administered for 2 min, and ischemia at 37°C was maintained for 30 min. At the end of the ischemic period, hearts were reperfused with KH for 30 min.

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 antioxidative 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 m*M* sucrose, 10 m*M* Tris-HCl, 1 m*M* EDTA, 0.5 m*M* dithiothreitol, and 0.1 m*M* 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 μ*M* cytochrome *c*, 71.4 μ*M* xanthine, 35.7 m*M* 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 H_2O_2 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% H_2O_2. 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 m*M* phosphate buffer pH 7, 0.5 m*M* EDTA, 0.25 U/ml glutathione reductase, 0.1 m*M* glutathione, 0.15 m*M* NADPH, 0.01% $NaHCO_3$, and 10–50 μg of protein enzyme. The reaction was initiated by adding 20 μl of 12 m*M* *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 discolored. 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 Bonferoni test was used to compare differences between the groups at every checked point.

RESULTS

Table 1 summarizes LVDevP 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 Cl-IB-MECA with and without their specific antag-

TABLE 1. PREISCHEMIC VALUES

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

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

onist (DPCPX or MRS1523) 24 h before the experiment. However, hearts that were perfused with CCPA or Cl-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 Cl-IB-MECA or control hearts were found (Fig. 2). At 30 min of reperfusion, LVDevP was 78 ± 3% and 55 ± 9% in CCPA- and Cl-IB-MECA-perfused hearts, respectively, versus 59 ± 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 Cl-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 ± 3.3% and 75 ± 4.6% in CCPA- and Cl-IB-MECA-injected hearts, respectively, versus 59 ± 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 ± 3.5 versus 23 ± 6 mm Hg, $p < 0.05$).

CF also improved (91 ± 21% and 78 ± 7% in CCPA- and Cl-IB-MECA-injected hearts versus 64 ± 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 Cl-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 Cl-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).

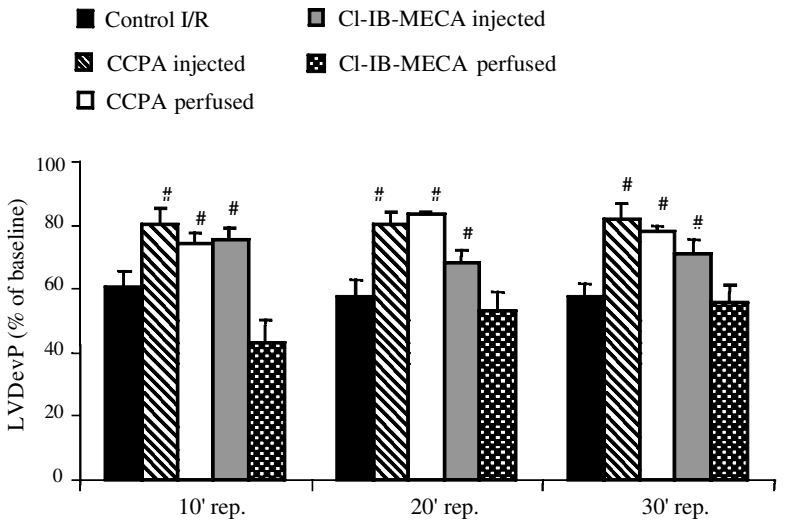

FIG. 2. LVDevP expressed as % of preischemic value in both modes of treatment with adenosine receptor agonists (perfused or injected). LVDevP at various time points following cardioplegic ischemia is presented. Values represent means ± SE. #$p < 0.05$ versus stabilization from the same group.

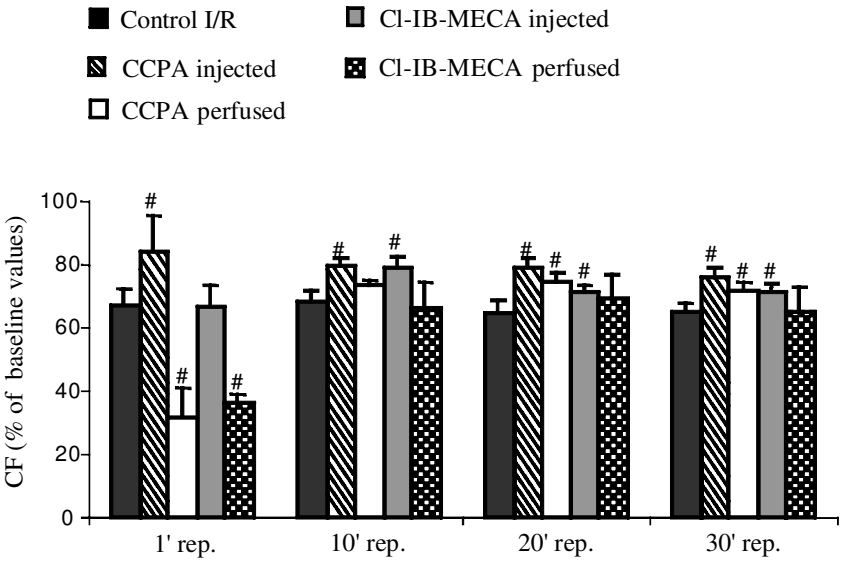

FIG. 3. CF rate expressed as % of preischemic value in both modes of treatment with adenosine receptor agonists (perfused or injected). CF at various time points following cardioplegic ischemia is presented. Values represent means ± SE. #$p < 0.05$ versus stabilization from the same group.

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 Cl-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 Cl-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-

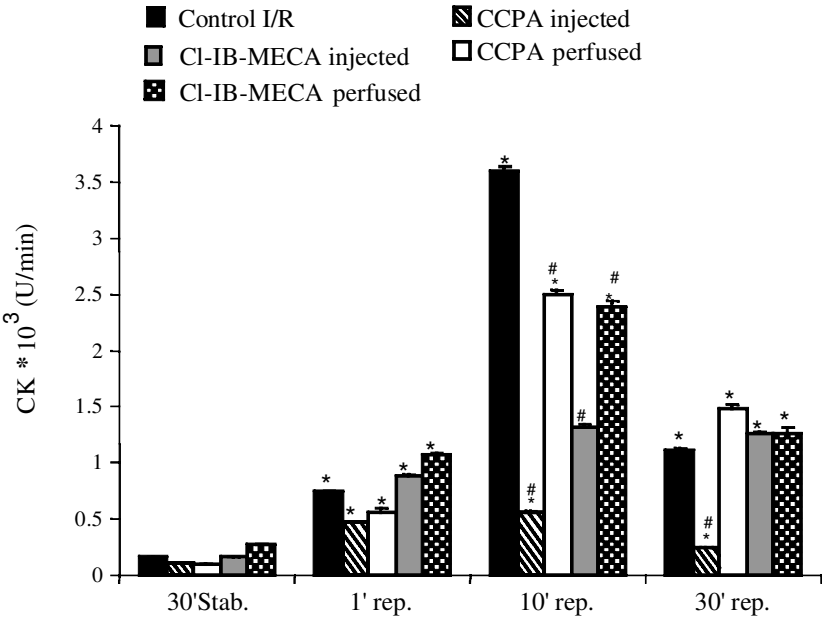

FIG. 4. Release of CK in both modes of treatment with adenosine receptor agonists (perfused or injected). Release of CK (U/min) to the coronary effluent during stabilization period and at various time points following cardioplegic ischemia is presented. Values represent means ± SE. #$p < 0.05$ versus stabilization from the same group. *$p < 0.05$ versus control I/R at the same time.

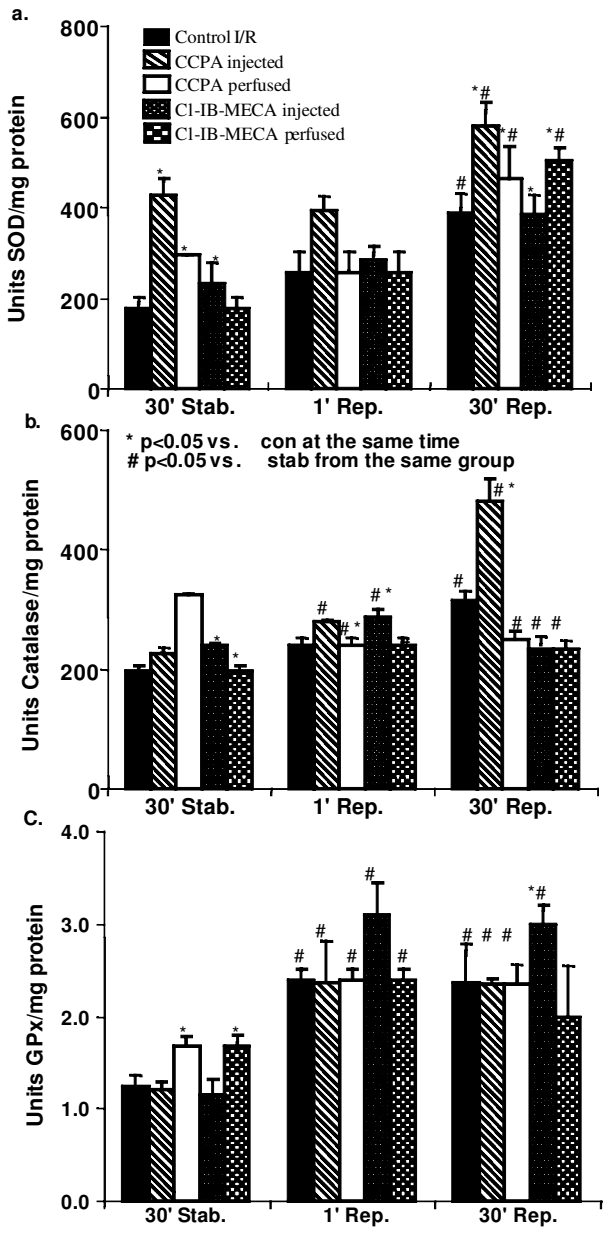

FIG. 5. Enzymatic activity of SOD (a), catalase (b), and GSH-Px (c) in hearts subjected to 30 min of ischemia followed by 30 min of reperfusion after treatments with adenosine receptor agonists. Antioxidant enzymatic activity was determined in myocardial extracts after 30 min of perfusion (stabilization), 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.

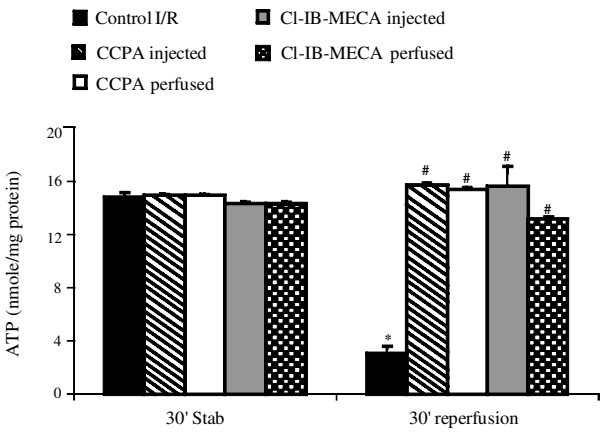

FIG. 6. ATP levels in hearts subjected to 30 min of ischemia followed by 30 min of reperfusion after treatments with adenosine receptor agonists. ATP was determined in myocardial extracts after ischemia and reperfusion. Values represent means ± SE ($n = 5$ in each group). #$p < 0.05$ versus stabilization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

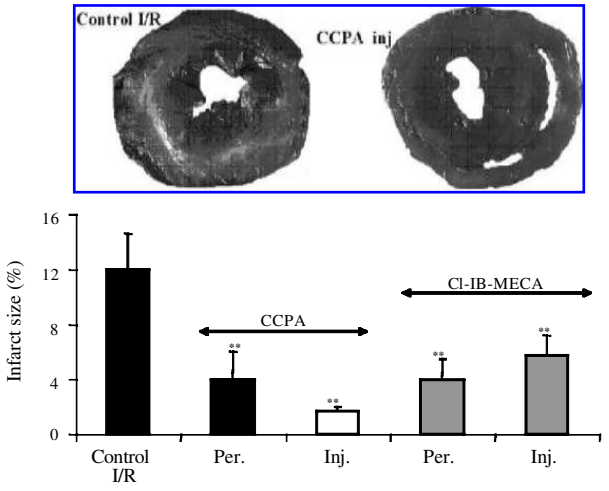

FIG. 7. Effect of I/R on myocardial infarction. Irreversible ischemia was determined by scanning the images of heart ventricular sections stained with TTC. Two representative images of the control I/R and CCPA-injected groups, revealing various degrees of myocardial ischemia, are presented. Hearts were 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.

trols than in the other groups. Mitochondria of many I/R treated hearts (Fig. 8A) were distorted with the swelling of matrix and contained electron–dense inclusions (calcium phosphate electron-dense deposits). Injection of hearts 24 h before the ischemic insult with A_1 receptor agonist CCPA significantly decreased mitochondrial damage. Well developed cristae were

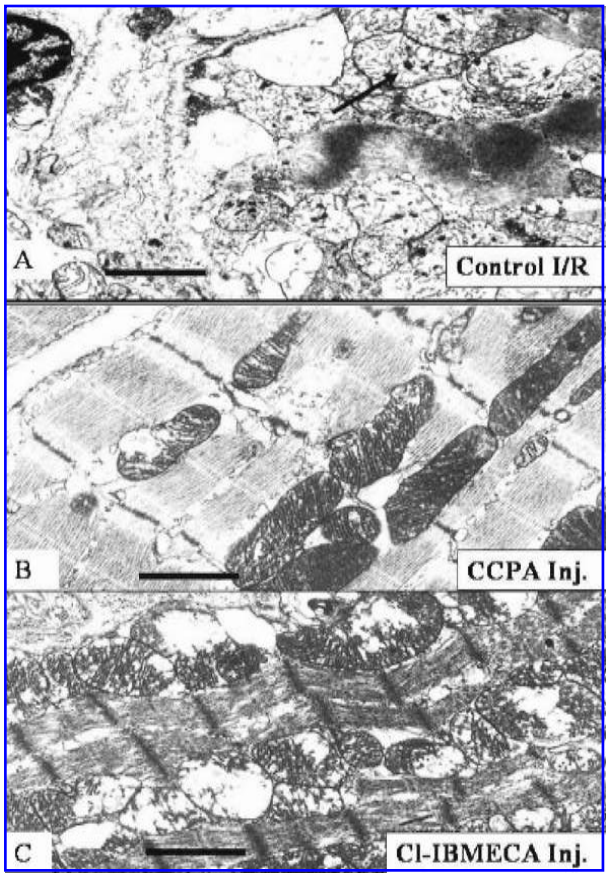

FIG. 8. Electron micrographs of hearts subjected to 30 min of ischemia followed by 30 min of reperfusion after treatments with adenosine receptor agonists. Electron micrographs of cardiac muscle of control I/R (**A**) and hearts injected with CCPA (**B**), and Cl-IB-MECA (**C**) are shown. Panel A demonstrates damaged cells in the control I/R heart. Mitochondria of I/R hearts contain a larger number of electron–dense deposits (arrows) than those of CCPA-injected or -perfused hearts (B). Protection with Cl-IB-MECA was less pronounced (C). The images represent sections performed in 5 rats/group. Bars = 1 μm.

seen in many organelles, and calcium deposits were less evident in the matrix of the majority of mitochondria (Fig. 8B). Protection by adenosine A_3 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 cardioplegic 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 cardioplegic 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 antioxidative 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_1 receptor agonist (delayed protection) afforded better myocardial protection than the A_3

TABLE 2. MORPHOMETRIC ANALYSIS OF PROTECTIVE EFFECTS OF ADENOSINE A_1 AND A_3 RECEPTOR ACTIVATION IN MYOCARDIAL CELLS AFTER I/R DAMAGE

	Mm	*Mv*	*MCa*	MF_r	MF_d
Control, no ischemia	28.2 ± 3.7	2.3 ± 2.8	—	56.3 ± 3.5	—
Control I/R	3.1 ± 4.6	15.3 ± 3.1	14.6 ± 2.5	12.1 ± 4.7	46.2 ± 5.6
CCPA injected	18.3 ± 2.8*	12.6 ± 2.2	4.5 ± 2.1*	38.1 ± 3.6*	17.3 ± 4.6*
Cl-IB-MECA injected	10.2 ± 4.3*	14.1 ± 1.8	8.4 ± 3.3*	28.6 ± 3.4*	27.1 ± 2.4*

Protective effects of adenosine A_1 and A_3 receptor activation were studied by the morphometric analysis of cardiac cells before (control, no ischemia) and after cardioplegic 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_r) and myofibrils with disruption (MF_d)]. Values are expressed as means ± SE.

*$p < 0.05$ versus control I/R.

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 A_1 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 A_1 receptor activation. Immediate A_1 and A_3 receptor activation elevated GSH-Px before ischemia, whereas delayed A_3 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 A_1 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 three enzymes in the CCPA-injected hearts was sufficient to lower ROS-induced cardiotoxicity. A_3 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 A_1 and A_3 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 the ischemic-reperfused myocardium (16, 37). These cardioprotective effects may be mediated by the activation of A_1 and A_3 receptors through the opening of ATP-sensitive potassium (K_{ATP}) channels, and consequent reduction of calcium overload (29, 36). A_3 receptor stimulation with Cl-IB-MECA, a highly specific adenosine A_3 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 A_1 and A_3 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-*N*^{6}-cyclopentyladenosine; CF, coronary flow rate; CK, creatine kinase; Cl-IB-MECA, 2-chloro-*N*^{6}-(3-iodobenzyl)adenosine-5′-*N*-methyluronamide; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; GSH-Px, glutathione peroxidase; H_2O_2, 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; $^{\bullet}O_2^-$, superoxide anion; ROS, reactive oxygen species; SOD, superoxide dismutase; TTC, 2,3,5-triphenyltetrazolium chloride.

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