Adenosine enhances cytosolic phosphorylation potential and ventricular contractility in stunned guinea pig heart: receptor-mediated and metabolic protection

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1Abteilung für Kardiologie und Pneumologie, Campus Benjamin Franklin, Charité Berlin, Berlin, Germany; 2Department of Physiology, Wayne State University School of Medicine, Detroit, Michigan; and 3Department of Anatomy, Physiology, and Genetics, Uniformed Services University of the Health Sciences, Bethesda, Maryland

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Schulze K, Duschek C, Lasley RD, Bünger R. Adenosine enhances cytosolic phosphorylation potential and ventricular contractility in stunned guinea pig heart: receptor-mediated and metabolic protection. J Appl Physiol 102: 1202–1213, 2007; doi:10.1152/japplphysiol.00245.2006.—Mechanisms of adenosine (ADO) protection of reperfused myocardium are not fully understood. We tested the hypothesis that ADO (0.1 mM) alleviates ventricular stunning by ADO A1-receptor stimulation combined with purine metabolic enhancements. Langendorff guinea pig hearts were stunned at constant left ventricular end-diastolic pressure by low-flow ischemia. Myocardial phosphate metabolites were measured by 31P-NMR, with constant left ventricular end-diastolic pressure by low-flow ischemia.

In addition, ADO is well known as a precursor for two major purine salvage pathways in the myocardium: the ADO kinase that rephosphorylates ADO to intracellular 5‘-AMP and the ADO desaminase-purine nucleoside phosphorylase-phosphoribosyl-pyrophosphate (PRPP) transferase system, which utilizes inosine (INO) and hypoxanthine for resynthesis of 5‘-AMP (26). These purine salvage pathways rebuild depleted cardiac adenylate pools effectively. Accordingly, it has been argued that metabolic enhancements by ADO help stabilize the cytosolic ATP pool and hence possibly the thermodynamic phosphorylation potential. Increased phosphorylation potentials could improve cardiomyocyte calcium control (21, 24, 27) and thus render the heart more tolerant of stress and ischemia-reperfusion injury (4, 23).

The present study attempts to distinguish between receptor-mediated signaling and direct metabolic effects of ADO in protecting against ventricular stunning, using a constant-pressure-perfused isovolumic guinea pig heart model. Perfusion medium was an oxygenated Krebs-Henseleit medium. Low-flow ischemia-reperfusion induced a moderate degree of left ventricular stunning with minimal ATP depletion, indicating the absence of myocardial infarction. Ventricular hemodynamic, energetic, and metabolic effects were measured, along with cell volume changes. The effects of therapeutic ADO (100 μM) were compared with those of selective ADO A1 agonist 2-chloro-N6-cyclo-pentyladenosine (CCPA; 0.01 μM), which was equipotent with ADO in terms of bradycardia but did not cause coronary dilation. Purine salvage pathway precursor INO (100 μM), nonspecific smooth muscle relaxant papaverine, and electrical pacing were used to assess the effects of bradycardia.
coronary hyperemia, and metabolically based purine salvage. Left ventricular systolic and diastolic contractility and ventricular power output, indexed by the heart rate (HR)-pressure product (RPP), were correlated with $^{31}$P-NMR-detectable free intracellular Pi, and the associated cytosolic phosphorylation potential. Myocardial IMP indexed the purine salvage, and glucose-6-phosphate (G6P) was the first and the rate-limiting intermediate of the NADPH-generating pentose phosphate pathway (PPP). To examine the possible role of myocardial potassium channels in ADO protection, we applied a saturating dose of the K$_{ATP}$ blocker glibenclamide in the presence of ADO.

METHODS

Langendorff Heart Perfusion and Low-Flow Ischemia-Reperfusion

All protocols were approved by the Institutional Animal Care and Use Committee at Charité Berlin and the Uniformed Services University of the Health Sciences. Hearts were isolated from female Hartley guinea pigs (350–400 g body wt) and perfused via the aorta at a constant physiological pressure (90 cmH$_2$O). The noncirculating hemoglobin- and cell-free Krebs-Henseleit perfusion medium contained all of the major blood electrolytes (in physiological mM concentrations: 116 NaCl, 3.5 KCl, 1.2 KH$_2$PO$_4$, 0.6 MgSO$_4$, 1.25 CaCl$_2$, 26 NaHCO$_3$, 5 glucose). Oxygenation and equilibration of the medium to pH 7.40–7.45 were provided by a gas mixture of 95% O$_2$-5% CO$_2$ at 37°C. The pulmonary artery was cannulated and connected to a narrow tube ending outside the NMR magnet. Coronary flow was measured continuously by a transit-time flow probe (Transonic Systems). Coronary effluent (1 ml/min) was diverted to a temperature-stabilized Clark-type oxygen electrode (Hugo Sachs) to measure PO$_2$. Myocardial oxygen consumption (MV$\dot{O}_2$) was calculated from the arteriovenous oxygen difference and coronary flow rate.

Steady-state protocols. For steady-state protocols, the following groups were used: 12 time controls, 10 with therapeutic ADO (100 $\mu$mol/l), 7 with selective ADO$_1$ agonist CCPA in noncoronary dilatary dose (0.01 $\mu$mol/l), 6 coronary hyperemia controls (coronary smooth muscle relaxation by 6 $\mu$mol/l papaverine), 6 with K$_{ATP}$ blockade by glibenclamide in the presence of 100 $\mu$mol/l ADO + 50 $\mu$mol/l glibenclamide, and 7 with metabolically based purine salvage (therapeutic 100 $\mu$mol/l INO, the deamination product of ADO).

In steady-state protocols, hearts were beating spontaneously at ~220 beats/min. A saline-filled latex balloon (Hugo Sachs) connected to a calibrated pressure transducer (Braun Melsungen) was placed into the left ventricle to record isovolumic end-diastolic pressure to a calibrated pressure transducer (Braun Melsungen) was placed into the left ventricle to record isovolumic end-diastolic pressure to a calibrated pressure transducer (Braun Melsungen). A saline-filled latex balloon (Hugo Sachs) connected to a narrow tube ending outside the NMR magnet. Coronary flow was measured continuously by a transit-time flow probe (Transonic Systems). Coronary effluent (1 ml/min) was diverted to a temperature-stabilized Clark-type oxygen electrode (Hugo Sachs) to measure PO$_2$. Myocardial oxygen consumption (MV$\dot{O}_2$) was calculated from the arteriovenous oxygen difference and coronary flow rate.

Time course protocols. For time course protocols, the following groups were used: 7 controls and 7 with therapeutic ADO (100 $\mu$mol/l).

In time course protocols designed to characterize the metabolic ischemia-reperfusion transitions in terms of ATP, intracellular P$_i$, and intracellular pH (pH$_i$) with and without ADO treatment, the Langendorff hearts were perfused without an intraventricular balloon. Intraventricular fluid accumulations were minimized by incisions in the left atrium and the mitral valve. In these experiments, 15 min of mild, low-flow (1 ml/min $^{-1}$g$^{-1}$) ischemia was followed by 30 min of reperfusion.

$^{31}$P-NMR Spectroscopy

In all steady-state protocols, noninvasive $^{31}$P-NMR spectra were acquired on an AMX400 WB pulsed Fourier transform spectrometer (Bruker) equipped with a 9.4-T superconducting magnet (Spectrospin). A 20-mm $^{31}$P probe (Bruker) with a 90° pulse length of 75 $\mu$s at 162 MHz was employed. The magnetic field was adjusted by the water proton-free induction decay. Partially saturated $^{31}$P spectra were accumulated by a radio frequency pulse width of 58 $\mu$s, resulting in a 70° tilt angle, a sweep width of 35 parts per million, 2,000 data points in the time domain, and a pulse interval of 3 s. Each spectrum was a time average over 96 scans, with total spectral acquisition time of 5 min. Spectra were processed with WIN-NMR software (Bruker). Zero filling to 4,000 data points and exponential multiplication of the data (5-Hz line broadening) was followed by Fourier transformation, automatic phasing, and baseline correction.

In the time course protocols, $^{31}$P-NMR scans were acquired in a Bruker wide-bore BZH 300 vertical magnet (7.05 T, 7.0 cm bore diameter) at a phosphorus resonance frequency of 121.5 MHz. Shimming was performed on a heart-sized glass sphere containing 2.5 ml of 2 M phosphate. 2-Methylene-diphosphonate (8.5 $\mu$mol/l) was placed inside the coil volume within a small glass sphere to allow the conversion of peak integrals into absolute amounts of metabolites. Hearts were placed in an NMR glass tube (25 mm outer diameter, 1 mm wall thickness; modified from Wilmad, Buena, NJ). A Helmholtz-type radio frequency transmitter and receiver coil (25 mm inner diameter, 23 mm height) made of 1.8-mm-wide copper strip were mounted at the glass tube. To reduce inductive and dielectric losses, the capacitance of the coil was divided by two 47-pF chip capacitors, and the “foil on edge” design was used, as previously described by Balaban et al. (2). The advantage of this design was that the resonance frequency of the tuned and matched coil shifted only ~0.35 MHz when loaded with saline. In addition, the half-line width of the resonance of a phosphorus phantom was 2 Hz, markedly better than that of commercial coils (>5 Hz). $^{31}$P-NMR spectra were collected over 5-min intervals as the sum of 176 single pulse acquisitions each. Acquisition parameters were as follows: size 2,000, sweep width 5,000 Hz, pulse width 40 ms (nominal 100-W amplifier), interpulse delay of 1.7 s, line broadening of 20 Hz for routine spectra, and 5 Hz for the phosphorus resonances. The Lorentzian line fit of the two P$_i$ resonances was performed by applying the GLINFIT program (version 870401 by Alex D Bain; Bruker Spectrospin Canada).

$^{31}$P-NMR Data Processing and Intracellular P$_i$

The following data were derived from each spectrum: area under the peaks of the external standard, total P$_i$, creatine phosphate (CP), $\beta$-ATP, and the chemical shifts relative to the CP peak of the intracellular phosphate and of the three ATP peaks. The $^{31}$P-NMR P$_i$ resonance showed as two peaks reflecting intracellular and extracellular P$_i$. The high spectral resolution allowed the discrimination between intracellular P$_i$ and extracellular P$_i$, using the partially overlapping twin resonance of the total P$_i$ and separating them analytically into two peaks. Peak integrals were converted to myocardial metabolite mass (amounts) by correcting for partial saturation, division by the standard peak area, and division by the dry mass of the heart.
Intracellular free concentrations of metabolites, indicated by brackets, were obtained by dividing the $^{31}$P-NMR-detectable metabolite masses by intracellular water volumes measured outside the magnet using radiolabeled mannitol as extracellular tracer (see below). pH$_i$ was estimated by applying the following equation for the chemical shift ($\delta$) of intracellular P$_i$ as a function of pH: pH$_i = 6.79 - \log[(6 - 5.75)/(3.25 - \delta)]$. A representative $^{31}$P-NMR spectrum is shown in Fig. 1.

**Cytosolic Phosphorylation Potential**

The metabolite ratio [ATP]/([ADP]+[P$_i$]), often referred to as phosphorylation potential (4, 18, 19, 22, 42), is the concentration term of the thermodynamic Gibbs free energy of ATP hydrolysis. Because the myocardium has abundant creatine kinase (CK), the value of this ratio could be estimated from the CK equilibrium as ([CP]/[H$^+$])/([Cr]/[P$_i$])x$K_{CK}$, where [H$^+$] is the intracellular H$^+$ concentration and $K_{CK}$ is the pH- and Mg$^{2+}$-dependent CK equilibrium constant, as detailed previously (5). The masses of myocardial ATP, CP, and P$_i$ and pH$_i$ were all obtained by $^{31}$P-NMR spectroscopy (see above). Creatine was measured enzymatically according to Bünger et al. (4).

The free cytosolic [ATP]-to-[ADP] ratio was calculated by use of the CK equilibrium in the form of ([CP]/[creatine])x[H$^+$]/$K_{CK}$). Free intracellular [P$_i$] was the intracellular P$_i$ mass divided by the radiochemically determined intracellular volume (ICV) (see below).

**ICV, Glycolytic Hexose, and Triose Phosphates and IMP as Index for Purine Salvage Pathway**

Myocardial extracellular and intracellular spaces were determined outside the magnet by infusing $^{[14]}$Cmannitol (50 $\mu$M, specific activity 59 Ci/mol; Amersham, Arlington Heights, IL) for 6 min followed by freeze-clamping the myocardium. Radioactivities in neutralized perchloric acid myocardial extracts were referred to the volume-specific activity of mannitol of arterial perfusate samples using a Searle Analytic 81 liquid scintillation system (Des Plaines, IL). Total tissue water was the difference between myocardial wet mass and myocardial dry mass obtained after 36 h of desiccation at 110°C. Total ICV was the difference between total tissue H$_2$O minus $^{[14]}$Cmannitol space.

To control for possible effects of the bradycardia due to ADO $\alpha_1$-receptor activation by ADO and CCPA, hearts in which glycolytic intermediates and glycolytic flux as well as 5'-IMP were measured were electrically paced at a rate of 270 beats/min. However, electrical pacing leads to distortions of the $^{31}$P-NMR heart spectra, making accurate intracellular metabolite and pH estimates impossible. Data showed that pacing to offset the 25–40% ADO/A$_1$ bradycardia did not greatly change MV$_3$ in reperfusion, indicating that the ADO/CCPA bradycardia per se was not the chief element in ADO protection of phosphorylation potential and ventricular contractility. It seemed therefore acceptable in this study not to electrically pace hearts when $^{31}$P-NMR measurements were performed.

**Glycolytic intermediates and glycolytic flux.** Perchloric acid extraction of frozen myocardium has been detailed elsewhere (4). All major glycolytic hexose and triose phosphates [G6P, fructose-6-phosphate, fructose-1,6-diphosphate, dihydroxyacetone phosphate, $\alpha$-glycerophosphate (estimated from $\alpha$-glycerophosphate dehydrogenase equilibrium), 3-phosphoglycerate, 2-phosphoglycerate, glyceraldehyde-3-phosphate, phosphoenolpyruvate], as well as pyruvate and lactate, were measured enzymatically on the day of extraction with the use of a dual-wavelength and dual-beam spectrophotometer (SLM-Aminco model DW2000). Glycolytic metabolic flux rate was measured radiochemically as the sum of $^{14}$CO$_2$ production from [U-$^{14}$C]glucose plus release of lactate + pyruvate (25).

**IMP.** Neutralized myocardial extracts (0.5 ml) were incubated for 30 min in 0.1 M triethanolamine (pH 7.6, 25°C) containing added nucleoside phosphorylase-xanthine oxidase (Boehringer Mannheim Biochemicals, Indianapolis, IN) plus 10 $\mu$M erythro-9–2-hydroxy-3-nonyl adenine-HCl (Burroughs Wellcome, Research Triangle Park, NC) to inhibit ADO deamination. During this 30-min incubation, preexisting purines (INO, hypoxanthine, xanthine) in the extracts were converted to urate, which provided the baseline absorbance for the IMP test at wavelength = 293 nm. Subsequently, the dephosphorylation and conversion of extract IMP to urate were started by adding 5 $\mu$l of alkaline phosphatase. The absorbance increase at 293-nm wavelength was proportional to the IMP content in the myocardial extract.

**Statistical Analyses and Curve Fitting**

Data are presented as means ± SD or ± SE as indicated. Student’s paired t-test was used to compare means within groups. A one-way ANOVA was used for between-group comparisons. The ANOVA included the Kolmogorov-Smirnov test for normality distribution, the Levene Median test for equal variance, and the least and honestly...
significant difference corrections for multiple comparisons. A two-tailed $P < 0.05$ was considered significant. For nonlinear fitting of equations, a purely descriptive polynomial regression was compared with a specific categorical logarithmic model, using the Gaussx 6.0/Gauss 5.0 algorithms (Aptech Systems). Residuals of the fitted equations were evaluated by Durban-Watson (DW) statistics for collinearity, serial errors, and model misspecifications, with heteroscedasticity routines to detect inconstant error variance.

RESULTS

ADO, Not CCPA, Protects Reperfusion Contractility

The 30-min low-flow ischemia imposed a moderate ischemic injury, which was sufficient to render the ventricle reproducibly stunned at 30-min reperfusion (Fig. 2). Stunning was evident from $\sim 20\%$ reduction in ventricular contractility ($dP/dt_{\text{max}}$, $dP/dt_{\text{min}}$, LVP; all $P < 0.05$). Also, the RPP indexing ventricular power and representing the major determinant of $MV_O_2$ was depressed $38\%$ in reperfusion. Accordingly, $MV_O_2$ decreased $22\%$ ($P < 0.05$). None of the treatments completely restored RPP or $MV_O_2$. In fact, $MV_O_2$ (in $\mu$mol $O_2\cdot$min$^{-1} \cdot g$ wet wt$^{-1}$) was not different between all groups in reperfusion: $1.34 \pm 0.22$ (control), $1.27 \pm 0.22$ (ADO), $1.20 \pm 0.18$ (CCPA), $1.21 \pm 0.21$ (papaverine), $1.06 \pm 0.12$ (ADO + glibenclamide), and $1.31 \pm 0.20$ (INO). Nevertheless, there were striking differences between treatment groups with respect to ventricular contractility (see below). The stunned hearts were slightly bradycardic ($170$ vs. $224$ beats/min; $P < 0.05$). The observed reperfusion losses in ventricular contrac-

Fig. 2. Effects of treatment with 100 $\mu$M ADO, 0.01 $\mu$M 2-chloro-$N^\prime$-cyclo-pentyladenosine (CCPA), ADO + glibenclamide (Glib), papaverine (pap), and inosine (INO) on heart rate (beats/min), coronary flow, left ventricular contractility ($dP/dt_{\text{max}}$ and $-dP/dt_{\text{min}}$), left ventricular pressure (LVP), and rate-pressure product (RPP) in ischemia-reperfused guinea pig hearts. Values are means $\pm$ SD ($n = 7–12$). Data refer to steady states of the preischemic period (light gray bars) and after 30 min of reperfusion (dark gray bars). For further details of protocols, see METHODS. $^*$ $P < 0.05$ preischemia vs. reperfusion within the same group (paired $t$-test), $^*P < 0.05$ vs. controls in preischemia or reperfusion.
tility and RPP reflected a delayed recovery of postischemic ventricular mechanics, the hallmark of myocardial stunning.

**HR and coronary flow.** In preischemic controls, spontaneous HR decreased 42% with ADO and 29% with CCPA, but this difference was not significant (P > 0.05, Fig. 2). Thus 0.1 mM ADO and 10 nM CCPA were equipotent in terms of bradycardia under our conditions. As expected, nonreceptor-linked purine salvage precursor INO and nongenomic smooth muscle relaxant papaverine did not affect HR. Papaverine and ADO but not INO more than doubled coronary flow at constant perfusion pressure. CCPA did not cause coronary dilation, indicating that the drug did not activate coronary A2a receptors in the concentration applied (10 nM). Thus CCPA selectively activated and nearly saturated the ADO A1 receptors of ventricular cardiomyocytes (Kd, 10 nM). Thus CCPA selectively activated and nearly saturated the ADO A1 receptors of ventricular cardiomyocytes (Kd, 10 nM). Thus CCPA selectively activated and nearly saturated the ADO A1 receptors of ventricular cardiomyocytes (Kd, 10 nM).

**Ventricular contractility and power.** In preischemia, none of the treatments appreciably changed dP/dt max or dP/dt min despite the bradycardia due to ADO and CCPA (Fig. 2). However, preischemic peak isovolumic LVP was increased with ADO (+20%) and CCPA (+36%) as well. Because all hearts were perfused at constant aortic pressure and LVEDP, the increase in LVP reflected increased isovolumic contractile force. In addition, the ADO bradycardia resulted in an ~30% decrease in the RPP, indicating depressed ventricular power output. Thus ADO increased isovolumic contractile force but depressed RPP in preischemia. CCPA, on the other hand, increased contractile force without depressing RPP in the preischemic hearts. Coronary hyperemia due to papaverine or perfusion with nonvasoactive, nonbradicardic INO did not affect ventricular contractility or force or RPP in preischemia.

In reperfused stunned hearts, ADO moderately decreased HR (−34%) while increasing all contractility indexes: LVP (+45%), dP/dt min (+39%), and dP/dt max (+12%). Compared with the preischemic controls, ADO fully preserved contractility [LVP, dP/dt min, dP/dt max to 119 ± 12%, 102 ± 6%, and 91 ± 7%, respectively (Fig. 2)]. It is noteworthy that ADO in reperfusion (not in preischemia) showed improved hemodynamics (LVP, dP/dt max, dP/dt min) not only vs. controls but also vs. all other treatments (all P < 0.05, except dP/dt max ADO vs. CCPA was not significant). CCPA was equipotent with ADO with regard to bradycardia also in the stunned hearts. However, unlike ADO, CCPA failed to effectively restore contractility. At 30-min reperfusion, there still was a decrease in dP/dt min (−14%; P < 0.05), dP/dt max (−16%; P < 0.05), and LVP (−5%; not significant) in the CCPA group. Thus CCPA was less effective in protecting reperfusion contractility than ADO.

Glibenclamide, a sulfonylurea with high affinity to cardiac microsomes (11), closes pharmacologically opened KATP in the myocardium but has no major effects on baseline action potential, HR, LVP, and RPP as well as coronary flow in guinea pig and dog hearts in vitro and in vivo, respectively (9, 44). We found that the drug did not significantly alter ventricular contractility and RPP in preischemic hearts treated with ADO. In contrast, it fully blocked ADO protection against ventricular stunning and deenergization in reperfusion (Fig. 2).

Treatment with the purine salvage precursor INO marginally (P > 0.05) protected postischemic dP/dt min and there were only minor effects on ventricular systolic contractility (LVP, dP/dt max). Pharmacological coronary hyperemia due to papaverine failed to protect both reperfusion contractility and RPP.

Because ADO treatment markedly reduces free Pi (see following paragraph), indexes of contractility were measured in an additional series of n = 4 hearts perfused with 0.6 mM phosphate (1/2 of the normal concentration) according to the steady-state protocol to exclude artificial errors caused by low Pi. The results are shown in Table 1. They indicate that Pi-changes per se do not change left ventricular hemodynamics.

**ADO and INO but not CCPA Decrease Cytosolic Free Pi and Preserve Energy Metabolism**

In preischemic hearts, ADO and CCPA raised the 31P-NMR-detectable ATP- and CP-concentrations substantially, mainly due to changes in ICV. Also creatine concentrations, measured enzymatically, increased ~45%, again reflecting the measured decrease in ICV.

Despite these volume changes, ADO lowered the concentration of free intracellular Pi relative to that of ATP (Table 2, Fig. 1). Although absolute [Pi] did not change, [Pi]/[ATP] decreased from 0.48 to 0.30 (−38%; P < 0.05). This Pi-lowering effect of ADO was not reproduced by selective ADO A1-receptor stimulation. Instead CCPA increased [Pi] by 2 mM, and [Pi]/[ATP] did not decrease. Because CCPA was equibradicardic with ADO, the data demonstrated that bradycardia per se was not the main cause for the Pi-lowering effect of ADO.

Also, coronary hyperemia due to papaverine did not lower [Pi] or [Pi]/[ATP] (Table 2). In addition, KATP blockade by glibenclamide did not abolish the Pi-lowering effect of ADO judged by the [Pi]-to-[ATP] ratio in preischemia. Only INO, which did not affect ventricular, coronary, or HR function, showed the Pi-lowering effect of ADO in preischemia. Together, these findings indicate that the Pi-lowering effect of ADO in the preischemic hearts was not mediated by ADO receptors or by KATP opening. Instead, it likely reflects metabolic enhancement due to purine salvage (26, 47).

In reperfused hearts, ATP and CP pools were only moderately decreased (~20%) relative to untreated preischemic controls. [Pi] and [Pi]/[ATP] were increased in all groups except those treated with ADO, CCPA, or INO. We also noted that ATP and CP concentrations were within the physiological range in all reperfused groups. Interestingly, in the presence of glibenclamide, the Pi-lowering effect of ADO was blocked. On the other hand, nonreceptor-linked purine (INO) had no such effect on Pi-lowering, indicating that the Pi-lowering effect of ADO in preischemia was not due to reaction of ATP or CP with Pi.

Table 1. Effect of reduced perfusate phosphate concentration on functional parameters (LVP, dP/dt max, dP/dt min) in ischemia-reperfused guinea pig hearts

<table>
<thead>
<tr>
<th>Protocol</th>
<th>LVP, mmHg</th>
<th>dP/dt max, mmHg/s</th>
<th>dP/dt min, mmHg/s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Perfusion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Perfusate phosphate (1.2 mM)</td>
<td>73 ± 12</td>
<td>2,470 ± 230</td>
<td>1,680 ± 190</td>
</tr>
<tr>
<td>Perfusate phosphate (0.6 mM)</td>
<td>76 ± 14</td>
<td>2,380 ± 200</td>
<td>1,720 ± 130</td>
</tr>
<tr>
<td>Reperfusion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Perfusate phosphate (1.2 mM)</td>
<td>60 ± 12</td>
<td>2,010 ± 200</td>
<td>1,230 ± 200</td>
</tr>
<tr>
<td>Perfusate phosphate (0.6 mM)</td>
<td>62 ± 14</td>
<td>1,950 ± 180</td>
<td>1,300 ± 150</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 4. Data refer to steady states of the preischemic period and after 30 min of reperfusion. LVP, left ventricular pressure; max, maximum; min, minimum. For further details of protocols, see METHODS.
Table 2. Effects of ADO, CCPA, ADO + glibenclamide, and INO on myocardial high-energy phosphates and free phosphate in ischemia-reperfused guinea pig hearts

<table>
<thead>
<tr>
<th>Protocol</th>
<th>[ATP], mM</th>
<th>[CP], mM</th>
<th>[Pi], mM</th>
<th>[Pi]/[ATP]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Preischemia</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>6.27±0.20</td>
<td>11.38±0.30</td>
<td>2.97±0.13</td>
<td>0.48±0.03</td>
</tr>
<tr>
<td>ADO</td>
<td>11.19±0.23*</td>
<td>19.45±0.33*</td>
<td>3.31±0.16</td>
<td>0.30±0.01*</td>
</tr>
<tr>
<td>CCPA</td>
<td>11.23±0.35*</td>
<td>20.96±0.65*</td>
<td>4.98±0.21*</td>
<td>0.45±0.03</td>
</tr>
<tr>
<td>Papaverine</td>
<td>6.05±0.25</td>
<td>11.59±0.31</td>
<td>3.08±0.24</td>
<td>0.51±0.03</td>
</tr>
<tr>
<td>ADO + glibenclamide</td>
<td>10.41±0.29*</td>
<td>17.76±0.87*</td>
<td>3.16±0.20</td>
<td>0.30±0.01*</td>
</tr>
<tr>
<td>INO</td>
<td>6.57±0.20</td>
<td>12.60±0.43</td>
<td>2.15±0.08*</td>
<td>0.33±0.02*</td>
</tr>
<tr>
<td><strong>Reperfusion</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>5.79±0.14</td>
<td>9.98±0.16†</td>
<td>3.72±0.21†</td>
<td>0.65±0.04†</td>
</tr>
<tr>
<td>ADO</td>
<td>10.04±0.34*</td>
<td>15.51±0.53*</td>
<td>3.13±0.08*</td>
<td>0.32±0.02*</td>
</tr>
<tr>
<td>CCPA</td>
<td>9.09±0.27†</td>
<td>15.47±0.29†</td>
<td>4.25±0.18†</td>
<td>0.47±0.02†</td>
</tr>
<tr>
<td>Papaverine</td>
<td>5.50±0.16†</td>
<td>9.26±0.57†</td>
<td>4.96±0.15†</td>
<td>0.90±0.02†</td>
</tr>
<tr>
<td>ADO + glibenclamide</td>
<td>7.88±0.27†</td>
<td>13.24±0.53*</td>
<td>3.79±0.21†</td>
<td>0.48±0.03†</td>
</tr>
<tr>
<td>INO</td>
<td>6.48±0.24†</td>
<td>11.43±0.47†</td>
<td>2.61±0.18†</td>
<td>0.41±0.04†</td>
</tr>
</tbody>
</table>

Values are means ± SE from the hearts of Fig 2. Values indicate free intracellular concentrations (brackets) as determined by 31P-NMR and radiochemically using the mean intracellular spaces according to Table 4. Data refer to steady states at the end of the normoxic period (preischemia) and to the postischemic steady state at 15–30 min (reperfusion). ADO, 100 μM adenosine; CCPA, 0.01 μM 2-chloro-N6-cyclopentyladenosine; INO, inosine. *P < 0.05 vs. the respective control group in preischemia or reperfusion (Bonferroni t-test). †P < 0.05 preischemia vs. reperfusion (paired t-test).

other hand, INO again decreased absolute [Pi] markedly (30%), confirming that metabolic enhancement by purine salvage can duplicate the Pi-lowering effect of ADO.

ADO, CCPA, INO, and papaverine all slightly raised the free [ATP]-to-[ADP] ratios in the preischemic control groups (Table 3). Interestingly, ADO did not raise [ATP]/([ADP]+[Pi]) in preischemia, but INO increased it substantially. In contrast, CCPA decreased [ATP]/([ADP]+[Pi]) 30% in preischemia. Blocking KATP with glibenclamide in the presence of ADO only slightly decreased [ATP]/([ADP]+[Pi]) by 16% (Table 3).

Reperfusion deenergized hearts, as evidenced by >40% decreases in cytosolic free [ATP]/[ADP] and [ATP]/([ADP]+[Pi]) in the control, CCPA, and papaverine protocols. Only ADO and INO effectively protected [ATP]/([ADP]+[Pi]) in stunned myocardium, reaching 81% of the level seen in the preischemic controls. Accordingly, ANOVA showed that, compared with the ADO treatment, all cytosolic phosphorylation potentials except those of the INO group were significantly reduced in reperfusion (all P < 0.05). The KATP blockade by glibenclamide completely blunted protection of [ATP]/([ADP]+[Pi]) in reperfusion (Table 3), comparing well with the blockade of inotropic protection under the same conditions.

**ADO Stimulates Glucose-6-Phosphate Accumulation and Myocardial Lactate Release**

G6P, substrate for metabolic flux through the NADPH-generating PPP, was similar in preischemic and in reperfused hearts.

Table 3. Effects of ADO, CCPA, glibenclamide, and INO on myocardial [ATP]-to-[ADP]-ratios and on cytosolic phosphorylation potentials in preischemic and postischemic stunned guinea pig hearts

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Free [ADP], μM</th>
<th>Free [ATP]/[ADP]</th>
<th>[ATP]/([ADP]+[Pi]), mM⁻¹</th>
<th>ΔGATP, kJ/mol</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Preischemia</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>52±2</td>
<td>121±4</td>
<td>42.1±3.1</td>
<td>−57.62±0.17</td>
</tr>
<tr>
<td>ADO</td>
<td>87±3*</td>
<td>130±3*</td>
<td>38.6±1.4</td>
<td>−57.63±0.12</td>
</tr>
<tr>
<td>CCPA</td>
<td>77±3*</td>
<td>146±4*</td>
<td>29.7±1.4*</td>
<td>−57.07±0.13*</td>
</tr>
<tr>
<td>Papaverine</td>
<td>43±1*</td>
<td>140±6*</td>
<td>46.4±3.6</td>
<td>−57.85±0.19</td>
</tr>
<tr>
<td>ADO + glibenclamide</td>
<td>100±2*</td>
<td>140±3*</td>
<td>33.4±1.2*</td>
<td>−56.96±0.08*</td>
</tr>
<tr>
<td>INO</td>
<td>47±2</td>
<td>140±3*</td>
<td>66.0±4.2*</td>
<td>−58.81±0.16*</td>
</tr>
<tr>
<td><strong>Reperfusion</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>81±2†</td>
<td>72±1†</td>
<td>19.8±1.1†</td>
<td>−55.65±0.13†</td>
</tr>
<tr>
<td>ADO</td>
<td>101±5*†</td>
<td>93±2†</td>
<td>34.2±1.0†</td>
<td>−57.33±0.14†</td>
</tr>
<tr>
<td>CCPA</td>
<td>94±3*</td>
<td>97±3*</td>
<td>23.1±1.3†</td>
<td>−56.40±0.11†</td>
</tr>
<tr>
<td>Papaverine</td>
<td>72±4*</td>
<td>78±4*</td>
<td>15.8±1.1†</td>
<td>−55.01±0.24†</td>
</tr>
<tr>
<td>Adenosine + glibenclamide</td>
<td>100±5*</td>
<td>80±4*</td>
<td>21.3±1.3†</td>
<td>−55.98±0.12†</td>
</tr>
<tr>
<td>INO</td>
<td>76±4†</td>
<td>86±3*†</td>
<td>33.8±3.0*†</td>
<td>−57.00±0.25†</td>
</tr>
</tbody>
</table>

Values are means ± SE from the hearts of Fig 2. Data refer to steady states at the end of the normoxic period and from 15 to 30 min after start of reperfusion. Free cytosolic [ADP] and [ATP]/[ADP] were calculated by use of the creatine kinase (CK) equilibrium in the following form: ([CP]/[Cr])/(H⁺/[K₅]), where [H⁺] is the intracellular H⁺ concentration, CP is creatine phosphate, Cr is creatine, and K₅ is the pH- and Mg⁺⁺-dependent CK equilibrium constant [see METHODS (4, 42)]. The concentration term of the Gibbs free energy (ΔGATP) is given by the metabolite ratio [ATP]/([ADP]+[Pi]), also called the phosphorylation potential. ΔGATP was estimated using ΔGATP (−30.1 to −30.4 kJ/mol, 37°C) according to Rosing and Slater (37) and measured intracellular pH and free [Mg²⁺]. *P < 0.05 vs. the respective control group in preischemia or reperfusion (Bonferroni t-test). †P < 0.05 within group preischemia vs. reperfusion (paired t-test).
controls (0.13 ± 0.01 mM). These concentrations are more than twofold the \( K_m \) of ~0.3 mM of G6P dehydrogenase, the rate-controlling enzyme of the PPP, indicating physiological substrate limitation of PPP flux. ADO treatment increased G6P levels in preischemia and reperfusion to 0.19 and 0.24 mM (Fig. 3), respectively (\( P < 0.05 \)), closer to the range of the \( K_m \) of G6P dehydrogenase, indicating increased PPP flux capacity. Also, CCPA increased G6P concentrations severalfold in reperfusion (0.39 ± 0.03 mM). Thus ADO and ADO \( A_1 \) agonism increased the antioxidant potential of the myocardium via the PPP.

Myocardial lactate release was minimal in control hearts in preischemia and reperfusion (0.04 ± 0.01 \( \mu \)mol/min \(^{-1} \)g wet wt\(^{-1} \)). Obviously, reperfusion and stunning did not greatly stimulate myocardial lactate export under the present conditions. Both ADO and CCPA stimulated lactate release in reperfusion to 0.07 ± 0.01 and 0.11 ± 0.3 \( \mu \)mol/min \(^{-1} \)g wet wt\(^{-1} \), respectively (\( P < 0.05 \)). Because lactate export is mediated by the electroneutral monocarboxylate-proton symporter (36), ADO treatment contributed to the removal of cytoplasmic protons in reperfusion, inducing a mild intracellular alkalization (Fig. 4D).

**ADO Stimulates Glycolytic Phosphate Binding, Not Glycolytic Flux**

In preischemic hearts, ADO increased the amount of \( P_i \) bound in glycolytic hexose plus triose phosphates (0.75 ± 0.05 mM vs. 0.53 ± 0.02 mM; \( P < 0.05 \)). Also, in reperfused hearts, ADO enhanced glycolytic phosphate fixation (1.84 ± 0.13 mM vs. 0.73 ± 0.06 mM; \( P < 0.05 \); Fig. 3). The enzymatically determined \( P_i \) binding was in excellent agreement with the reperfusion \(^{31} \)P-NMR \( P_i \) data of Fig. 4, in which the difference in \( P_i \) mass of ~3–4 \( \mu \)mol/g dry wt between control and ADO hearts was equivalent to a [\( P_i \)] decrease of ~1 mM. Similarly, Table 2 indicates an ADO-induced decrease in [\( P_i \)] of ~0.6 mM in reperfusion.

Despite enhanced \( P_i \) fixation by glycolytic intermediates, ADO only marginally stimulated glycolytic metabolic flux by ~15% (not significant) in any of the three protocol phases (preischemia, ischemia, reperfusion). In four hearts subjected to the mild ischemia of the time course protocol, the following glycolytic flux rates (\( \mu \)mol \( C_3 \)-min\(^{-1} \)g wet wt\(^{-1} \), ADO vs. control) were obtained: preischemia: 0.83 ± 0.06 vs. 0.70 ± 0.02 (not significant), ischemia: 1.10 ± 0.10 vs. 0.97 ± 0.09 (not significant), and reperfusion from 15 to 30 min: 0.80 ± 0.03 vs. 0.69 ± 0.02 (not significant). The ischemic increases in glycolytic flux of ~30% or 0.3 \( \mu \)mol \( C_3 \)-min\(^{-1} \)g wet wt\(^{-1} \) were minor compared with the maximum glycolytic flux capacity of the guinea pig heart of 7.5 \( \mu \)mol \( C_3 \)-min\(^{-1} \)g wet wt\(^{-1} \) in anoxia (25).

**ADO, Not CCPA, Stimulates 5’-IMP Formation**

IMP, a physiological adenylyl precursor, is an index of purine salvage activity via the PRPP-linked pathway, which is known to stabilize and/or replenish the myocardial ATP pool in postischemic and other stress conditions (46). IMP content was doubled by ADO in preischemia (0.10 ± 0.02 vs. 0.05 ± 0.01 \( \mu \)mol/g dry wt; \( P < 0.05 \)). However, CCPA did not affect IMP levels, indicating that CCPA is a poorly metabolizable ADO analog. Also, in reperfusion, ADO significantly increased IMP contents (0.14 ± 0.02 vs. 0.10 ± 0.01 \( \mu \)mol/g dry wt; \( P < 0.05 \)). Again, CCPA had no effect (0.07 ± 0.04 vs. 0.10 ± 0.01 \( \mu \)mol/g dry wt). This indicated that ADO \( A_1 \)-receptor agonism alone does not stimulate purine salvage, at least not in the absence of exogenous purines. On the other hand, ADO treatment intensified purine salvage and thus likely contributed to stabilization of myocardial ATP in reperfusion (Table 2, Fig. 4).

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**Cytosolic Phosphorylation Potential and Ventricular Contractility Tightly Correlate in Reperfused Myocardium Treated by ADO**

Figure 5 depicts observed correlations between systolic and diastolic contractility indexes and the \(^{31} \)P-NMR-estimated cytosolic phosphorylation potentials at 30 min reperfusion. Ignoring the INO group, the LVP and dP/d\( t \)max data plotted as a function of the reperfusion [ATP]/([ADP][\( P_i \])] show a curvilinear relationship with a very high polynomial regression coefficient (\( R^2 = 0.85 \) and 0.81, respectively). In the separate INO group, ventricular contractility only weakly increased with [ATP]/([ADP][\( P_i \])]. Correlation between dP/d\( t \)max and [ATP]/([ADP][\( P_i \])] was less definitive (\( R^2 = 0.70 \)).
The $R^2$ values deteriorated to $\leq 0.5$ when the contractility indexes were plotted against the individual components of the cytosolic phosphorylation potential (free [ATP], free [Pi], free [ATP]/[ADP]). On the other hand, a logarithmic and more physiological model that included the INO data confirmed the conclusions from the polynomial model. A nonlinear logarithmic model covered the entire data set ($N = 47$):

$$y = a_0 + a_1 \times \log\left(\frac{\text{[ATP]}}{\text{[ADP]} \times \text{[Pi]}}\right) + a_2 \times \text{CAT}.$$ 

This model included a constant $a_0$ and a categorical variable (CAT), which was set at CAT = 0 = no INO treatment or CAT = 1 = INO treatment, to account for the distinct effects of INO as a purine salvage precursor. Fitting statistics were as follows: 1) $y = dP/d_{\text{min}}$ had $R^2 = 0.81$ and DW of 2.08; 2) $y = dP/d_{\text{max}}$ had $R^2 = 0.78$ and DW = 1.8; 3) $y = \text{LVP}$ had $R^2 = 0.84$ and DW = 2.1. Because the fitted coefficient $a_2$ for the CAT variable was at $P < 0.005$ for all $y$ values, it confirmed the polynomial results and demonstrated that the efficacy of INO to restore reperfusion contractility was significantly lower than that of ADO. In

![Graphs showing ATP, CP, Pi, pH changes during ischemia and reperfusion](image)

Fig. 4. $^{31}$P-NMR detectable myocardial masses ($\mu$mol/g dry wt) of ATP (A), creatine phosphate (CP; B), free intracellular Pi (C), and intracellular pH (D) in glucose (circles) and in glucose + 100 $\mu$M ADO-perfused guinea pig hearts (triangles). After the initial normoxic perfusion, hearts were subjected to a 15-min low-flow global ischemia (1.0 ml \text{g}^{-1} \text{min}^{-1}) followed by 30 min of reperfusion. Data are means $\pm$ SE ($n = 7$). * $P < 0.05$ by one-way ANOVA relative to respective time controls.

Fig. 5. Scatter plots of reperfusion LVP (left) and $-dP/d_{\text{min}}$ (right) vs. the cytosolic phosphorylation potential, [ATP]/[ADP][Pi], in Langendorff guinea pig hearts stunned by low-flow ischemia-reperfusion. Data refer to the steady state at 30 min of reperfusion. • Controls; ○ ADO; △ CCPA; ▽ ADO + glibenclamide; ◦ papaverine; ▼ INO. Lines depict 4th-degree polynomial on the pooled data from all groups ($n = 41$), excluding the INO group.
addition, because the DW statistics proved close to the ideal value of 2.0 for all y values of the logarithmic model, there was no evidence that the model had serial errors or that key predictor variables were not included, which strengthens confidence in the model’s adequacy. When its predictive power for preischemic hearts was tested, preischemic [ATP]/([ADP][Pi]) was essentially correctly predicted when using the dP/dt values from the control and the INO groups. However, with the use of preischemic LVP values, the model underestimated preischemic [ATP]/([ADP][Pi]) by 34% (P < 0.05). We also tested the impact of the individual components of the phosphorylation potential (free [Pi], [ATP], free [ATP]/[ADP]) in reperfusion as predictors of the y values in the logarithmic model. We found that R^2 values deteriorated to below 0.5 and that the DW statistics became significant at ~1.1, indicating model misspecification, serial errors, and/or missing predictor variables.

**ADO and CCPA Decrease ICV**

Normoxic ICV of the Langendorff hearts was 0.37 ± 0.003 ml/g wet wt or 3.36 ± 0.11 ml/g dry wt, comparing well with ICVs for working guinea pig hearts (2.91 ± 0.10 ml/g dry wt) (6) but being somewhat higher than those from Langendorff rat hearts using the \(^1\)H/\(^{15}\)N-MR technique (1). Electrical pacing increased ICV by 10–30% (P < 0.01), whereas ADO decreased ICV 22% in paced and 43% in spontaneously beating hearts (Table 4). As an unrelated control, 5 U/L insulin had no measurable effect on ICV.

Reperfusion produced a 25% decrease in the ICVs of paced hearts (P < 0.01). The reperfusion ICVs of phosphorylase a pig hearts compared well with those of rat hearts (1). ADO and CCPA decreased ICV by 30% in reperfusion regardless of pacing (P < 0.05). For INO and papaverine protocols, ICVs were not measured. It was assumed that nonreceptor-linked INO and nonspecific smooth muscle relaxant papaverine did not alter cell volume, as these agents have no known affinities to cell volume regulatory A1/A3 purinoreceptor signaling pathways (for review, see Ref. 17). Because glibenclamide did not block A1 or A2a signaling, it was reasonable to assume that the ICV in the ADO + glibenclamide group was identical to ADO-perfused hearts.

Together, ADO and selective A1 purinoceptor stimulation consistently decreased ICV in preischemia and in reperfusion, independent of the prevailing HR.

**DISCUSSION**

The major findings of this study may be summarized as follows. Continuous ADO infusion (0.1 mM) of the stunned Langendorff guinea pig heart effectively protected ventricular contractility in reperfusion along with, but not at the expense of, the cytosolic phosphorylation potential (Table 3, Fig. 5). The selective A1 agonist CCPA (0.01 μM) was equiproteric with ADO yet only weakly protected reperfusion contractility and phosphorylation potential. Metabolic purine salvage (0.1 mM) by INO was equipotent with ADO in terms of preserving the reperfusion phosphorylation potential but less effective than ADO in protecting ventricular contractility. The \(K_{\text{ATP}}\) channel blocker glibenclamide abolished ADO protection of postischemic contractility and phosphorylation potential. Finally, although ADO failed to stimulate glycolytic flux, it produced G6P accumulation, enhanced glycolytic phosphate fixation, stimulated purine salvage, and augmented myocardial lactate release, associated with a mild intracellular alkalization. Obviously, the mechanisms of protecting the stunned myocardium by exogenous ADO are multifactorial and include activation of purine salvage and increased PPP flux potential combined with ADO receptor-linked signaling.

This study demonstrates a definitive proportionality between ADO-enhanced cytosolic phosphorylation potential and improved ventricular contractility in a Langendorff-perfused guinea pig heart stunned by low-flow ischemia. This ADO protection of reperfusion contractility occurred, however, at depressed ventricular RPP. With respect to [ATP]/([ADP][Pi]), ADO cardioprotection resembled that by metabolic inotropes such as pyruvate (23); both agents reduce ventricular dysfunction in reperfusion along with, but not at the expense of, the phosphorylation potential or its indexes (4). Statistical modeling
revealed that the individual elements of the phosphorylation potential (free intracellular [Pᵢ], free [ATP]/[ADP], [ATP]) were poor predictors of reperfusion contractility compared with the full [ATP]/([ADP]+[Pᵢ]) ratio (Fig. 5), consistent with the fact that the cellular calcium-handling ATPases are driven by [ATP]/([ADP]+[Pᵢ]) and not by [ATP] alone as the chief cellular energy source. Indeed, we previously reported that increased [ATP]/([ADP]+[Pᵢ]) was associated with improved sarcoplasmic reticulum calcium handling and increased calcium transients in isolated cardiomyocytes or intact perfused hearts (21, 24, 27).

However, in distinct contrast to the exclusively metabolically based interventions with INO or pyruvate, ADO exerted both metabolic and receptor-mediated effects: ADO stimulated purine salvage via the PRPP pathway and likely also via the ADO kinase (15). Control experiments with nonspecific smooth muscle relaxant papaverine revealed that coronary hyperemia per se did not protect against postischemic stunning or deenergization (Tables 1 and 3). Associated with A₁ receptor agonism by CCPA or ADO was G6P accumulation into the range of the Kₘ of the G6P dehydrogenase, which increased the potential for PPP-dependent NADPH formation and hence likely strengthened myocardial reactive oxygen species (ROS) tolerance in the presence of ADO or CCPA (32). In isolated perfused mouse hearts injured by 20 min of stop-flow ischemia-reperfusion, a similar combination of metabolic purine salvage with ADO receptor signaling was observed during ADO protection (35).

Because ADO only marginally stimulated glycolytic flux, yet substantially enhanced cytosolic [ATP]/([ADP]+[Pᵢ]), we suggest that ADO protected mitochondrial function and possibly also reduced the acute cytosolic energy requirements. The correlation between the phosphorylation potential and reperfusion ventricular contractility proved to be particularly strong at \( R^2 = 0.80–0.85 \). Because of the direct proportionality between the cytosolic [ATP]/([ADP]+[Pᵢ]) and ventricular function in energy-deprived conditions including reperfusion (4, 14, 20, 24, 28), our data suggest that the cytosolic phosphorylation potential should be a promising target for clinical pharmacology. From this result and the fact that the ADO did not noticeably alter the phosphorylation potential or the dP/dt indexes in preischemia (Tables 1 and 3), it must have exerted its beneficial effects primarily during ischemia and throughout reperfusion as well (45). It is thus tempting to suggest that clinical ADO applications with respect to cardioprotection could include prophylactic use of ADO in cardiac surgical procedures. It could also be of utility in the cardiology setting of acute myocardial ischemic conditions, especially when ischemia is mild and without infarct, for example, in thrombolytic coronary revascularization, coronary angioplasty, and stenting techniques (43).

Superior Antistunning Efficacy of ADO Relative to Adenosine A₁-Receptor Agonism or Metabolic Purine Salvage

Guinea pig and rat ventricular cardiomyocytes have surface ADO A₁ receptors with similar density (40). We observed that 10 nM CCPA and 100 μM ADO were equally bradycardic in the guinea pig hearts, indicating that A₁ signaling occurred with comparable intensity in both groups. Despite this apparent A₁-receptor saturation [dissociation constant for A₁ agonist binding is only 2–3 nM (40)], CCPA proved much less effective than ADO in protecting the reperfused heart energetically and inotropically (Figs. 2 and 5). This result also showed that antistunning protection by ADO could not be explained by bradycardia (A₁-receptor signaling) alone. In addition, unlike ADO, CCPA did not lower cytosolic free [Pᵢ] in reperfusion, which explains why CCPA failed to protect reperfusion [ATP]/([ADP]+[Pᵢ]) (Table 3) along with contractility (Fig. 5). Also, unlike ADO, CCPA did not raise myocardial IMP levels, indicating no stimulation of the energetically beneficial purine salvage by selective A₁ agonism.

As for the hemodynamic effects of INO compared with ADO, INO had no chronotropic or inotropic effects in preischemia (Fig. 2 (16)), demonstrating negligible affinity to the myocardial A₁ receptors in the guinea pig heart. On the other hand, exogenous purines can supply replenishment of the myocardial ATP pool postschemically mediated by anabolic purine salvage pathways (39). Because ADO raised myocardial IMP, the INO-hypoxanthine-PRPP-transferase pathway was boosted and most likely also the ADO kinase pathway (34). We also found that INO perfusion, much like ADO treatment, decreased cytosolic free [Pᵢ] in both preischemia and reperfusion (Table 2), resulting in considerable increases in the phosphorylation potentials (Table 3).

As for the cardioprotective efficacy of INO, it was important to realize that INO and ADO were equipotent with respect to the phosphorylation potential (Table 3, Fig. 5). However, unlike ADO, INO did not increase contractility and also did not cause bradycardia or coronary hyperemia [Fig. 2 (16)]. These data again confirmed that the ADO enhancement of the phosphorylation potential was likely not caused by the associated bradycardia. Similarly, CCPA bradycardia decreased, not increased, the phosphorylation potential in preischemia (Table 3).

INO was definitely less effective than ADO in protecting reperfusion contractility. Therefore, metabolic enhancement due to INO-driven purine salvage alone was not sufficient to optimally protect or restore ventricular contractility in reperfusion. A plausible but hypothetical explanation is that ADO receptor-mediated signaling could have improved energy-force coupling in reperfusion, whereas INO solely stimulated metabolic purine salvage.

Glibenclamide Blocks Adenosine Cardioprotection

We observed a complete blockade of cardioprotection by ADO in the presence of 50 μM glibenclamide. Already lower concentrations of glibenclamide fully blocked both reconstituted and intact KᵢATP channels (33, 40). Also Yao and Gross (44) demonstrated in in vivo dog hearts that glibenclamide abolished the protection against stunning afforded by the A₁ agonist CCPA. In contrast, Ford et al. (10) found no inhibition of functional protection by glibenclamide in working rat hearts. However, the protocol of Ford et al. and the Yao and Gross stunning protocol differ from ours and in crucial aspects. Ford et al. used the high ATP turnover-isolated working rat heart and not the resting working dog heart or the nonworking Langendorff guinea pig heart. In addition, the severe stop-flow ischemia-reperfusion protocol applied by Ford et al. greatly depleted myocardial ATP. We observed in a normothermic
30-min stop-flow guinea pig heart a 44% ATP reduction, associated with a large increase in free [Mg^{2+}] to above 1 mM. Millimolar free [Mg^{2+}] reduces the affinity of glibenclamide to the K_{ATP} (33). Therefore, it is conceivable that glibenclamide was not effective under the Ford et al. conditions.

Our low-flow ischemia-reperfusion protocol created only a mild intracellular acidosis of \( \sim \) pH 7.0. (Tables 2 and 3, Fig. 4). Similarly, ischemic glycolytic flux increased only ~30%, much below its ~650% reserve (25). Also, cardiac ATP was only mildly reduced. In addition, by \(^{31}\)P-NMR spectroscopy, the free intracellular magnesium concentration can be estimated from the chemical shift of the \( \alpha\)- and \( \beta\)-resonances of ATP. Applying this estimation procedure on our data, an increase of free [Mg^{2+}] of 0.1 mM can be noticed, far below the concentration required to reduce the affinity of glibenclamide to the K_{ATP}. As in our protocol, Yao and Gross (44) also imposed an only modest ischemic stress.

**Sarcolemmal K\(_{ATP}\) Opening as an Element in Adenosine Cardioprotection**

In reperfusion, ADO increased the cytosolic phosphorylation potential in conjunction with enhanced ventricular contractility. This finding cannot be explained by opening of mitochondrial K_{ATP}, as suggested earlier (15, 29, 35, 38). The reason is that opening of mitochondrial K_{ATP} would imply a decrease in the mitochondrial membrane potential. Because the latter directly determines the cytosolic phosphorylation potential (8, 18, 19), a fall, not an increase, in measured [ATP]/([ADP][Pi]) should be the result.

The possibility remains that ADO opens the sarcolemmal K_{ATP} in reperfusion, although ADO does not affect the sarcolemmal K_{ATP} in preischemia (40). This would facilitate electric K\(^+\) export and sarcolemmal hyperpolarization during recovery, shorten the action potential duration (9), reduce the open probability of the voltage-sensitive calcium channel, and reduce net calcium influx. The reduced cellular calcium load could help stabilize [ATP]/([ADP][Pi]), as shown in unstimulated Langendorff hearts vs. high-performing working hearts (6). Because the sarcolemmal K_{ATP} has a high affinity to glibenclamide, ADO opening of the channel would be reversed by the blocker and thus abolish ADO enhancement of [ATP]/([ADP][Pi]) in reperfusion. The observed glibenclamide-induced decrement in [ATP]/([ADP][Pi]) was directly matched with decreases in ventricular contractility, LVP, and dP/dt\(_{min}\) in particular (Fig. 5).

We can only speculate about the exact molecular mechanism(s) underlying the failure of ADO to protect [ATP]/([ADP][Pi]) in the presence of glibenclamide during reperfusion. One possibility is that ROS were involved. Already mild ischemia-reperfusion transitions produce bursts of reactive oxyradicals in the guinea pig heart (3). Opening of the sarcolemmal K_{ATP} protects against ROS injury in terms of ventricular function and cardiac ATP stores (12, 31).

ROS could oxidize essential sulfhydryl groups on the Ca\(^{2+}\)-handling ATPases, the sarcoplasmic reticulum calcium ATPase in particular, which could limit the cardiomyocytes' ability to rapidly lower cytosolic calcium in diastole, which in turn would reduce dP/dt\(_{min}\). It has long been known that stunned myocardium has a decreased myofilament Ca\(^{2+}\) sensitivity (13), which could well result in impaired energy-force coupling. However, energetically more detrimental would be ROS damage to the mitochondrial compartment, e.g., inactivation of the aconitase of the Krebs cycle (41) or impairment of the electron transport chain due to cytochrome c dissociation from the inner membrane. Such mitochondrial damage could depolarize the mitochondrial membrane, translating into decreased or unstable extramitochondrial (cytosolic) [ATP]/([ADP][Pi]) (8, 19). Lasley and colleagues (32) found that CCPA decreased the cellular ROS activity in rat cardiomyocyte ischemia-reoxygenation. This CCPA effect was blocked by glibenclamide, supporting the possibility that K_{ATP} opening has an antioxidant element.

We further observed that both ADO and CCPA raised reperfusion myocardial G6P concentrations into the range of the K_m = 0.3 mM of the G6P dehydrogenase. This implies increased PPP flux potential and hence cytosolic NADPH availability for GSSG reduction, which is critical for detoxification of intracellular ROS. Although we did not measure NADPH or glutathione redox ratios, which would provide much stronger evidence for increased antioxidant activity, the observed ADO protection of the reperfused guinea pig heart might have an antioxidant element, linked to or mediated by the ADO A_1 receptor, due to increased G6P accumulation and PPP flux potential.

**Conclusions**

The main results demonstrated 1) that ADO was more effective than ADO A_1 agonist CCPA in protecting against posts ischemic deenergization and ventricular dysfunction and 2) that ADO was also superior to metabolic enhancement by INO, as the latter did not effectively protect posts ischemic contractility, although myocardial phosphorylation potential was protected. This study does not argue against other mechanisms, i.e., A_3-receptor-mediated effects, playing a role in ADO cardioprotection. Together, our results suggest that cardiac stunning may be associated with defects in energy state and energy-force coupling, which appear to respond favorably to the combination of receptor-mediated, antioxidant, and purine-enhancing features of exogenous ADO.

**GRANTS**

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METABOLIC AND RECEPTOR-LINKED ADENOSINE CARDIOPROTECTION


