Protection From Reperfusion Injury After Cardiac Transplantation by Inhibition of Adenosine Metabolism and Nucleotide Precursor Supply

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Background—Adenosine (Ado) triggers numerous protective mechanisms in the heart that may attenuate ischemia-reperfusion injury in cardiac grafts. We aimed to establish whether sustained increase in endogenous Ado production by the combined application of Ado metabolism inhibitors and nucleotide precursors attenuates reperfusion injury in transplanted hearts.

Methods and Results—Rat hearts were collected after the infusion of St Thomas’ Hospital cardioplegic solution, stored at 4°C for 4 hours, and heterotopically transplanted into the abdomen of recipient rats. A solution containing Ado deaminase inhibitor erythro-9(2-hydroxy-3-nonyl)adenine, Ado kinase inhibitor 5′-aminoadenosine, and nucleotide precursors adenine and ribose was administered at the time of reperfusion in the treated group, whereas saline was administered to control animals. After 1 or 24 hours, mechanical function of the transplanted hearts was evaluated in an ex vivo perfusion system followed by the determination of myocardial ATP with related metabolites and measurement of the activity of neutrophil-specific enzyme myeloperoxidase in cardiac homogenates. After 24 hours of reperfusion, maximum left ventricular developed pressure increased from 87.0±6.8 mm Hg (mean±SEM) in controls to 118.1±8.2 mm Hg in the treated group (P<0.05), ATP increased from 11.0±0.8 μmol/g dry wt in controls to 15.1±1.2 μmol/g dry wt in the treated group (P<0.01), and myeloperoxidase activity decreased from 2.23±0.60 U/g wet wt in controls to 0.58±0.12 U/g wet wt in the treated group (P<0.001). No differences in cardiac function, ATP, or myeloperoxidase activity were observed between the treated group and controls after 1 hour of reperfusion.

Conclusions—The administration of Ado metabolism inhibitors with nucleotide precursors causes a sustained increase in endogenous Ado production and exerts a potent protective effect against reperfusion injury in transplanted hearts. Improved cardiac function and elevated ATP concentration were accompanied by complete amelioration of neutrophil infiltration in treated hearts, suggesting that reduction in postischemic inflammation could be an important mechanism of this protective effect. (Circulation. 2001;104[suppl I]:I-246-I-252.)

Key Words: adenosine ■ transplantation ■ reperfusion ■ inflammation ■ leukocytes

Adenosine (Ado), a catabolite of ATP, has long been recognized as a potent cardioprotective agent. This effect is a consequence of a wide spectrum of receptor-mediated and metabolic activities of Ado, such as coronary vasodilatation, antagonism of catecholamine effects, inhibition of thrombocyte aggregation, inhibition of neutrophil adhesion, free radical formation, antagonism of tumor necrosis factor-α effects, and induction of preconditioning.1–3 All these activities indicate that Ado, Ado receptor agonists, or compounds that increase Ado concentration could be valuable pharmacological tools in a variety of clinical situations associated with cardiac ischemia. Increased posts ischemic myocardial performance was demonstrated experimentally when exogenous Ado was applied either before ischemia or during reperfusion.4–7 Reduction in the infarct size was reported when Ado was used clinically as an adjunctive therapy in myocardial infarction,8 and benefits were also demonstrated in a phase II clinical trial of Ado in patients undergoing CABG.9 Ado is of special interest in the field of cardiac transplantation due to its combined cytoprotective, immunosuppressive, and anti-inflammatory effect. A major problem with direct administration of exogenous Ado is its very short half-life, which is <1 second in human blood.10 To maintain therapeutic concentration, it is necessary to infuse Ado continuously, preferably via the intracoronary route. Elevation of endogenous Ado through the administration of precursors or inhibitors that affect Ado metabolism has

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been proposed as alternative therapeutic strategy. This approach provides the opportunity to achieve the highest concentration at the target sites: in the vicinity of cardiac cells. Increased endogenous Ado production has been proposed to account for the cardioprotective effect of the purine precursor 5-amino-4-imidazole carboxamide (AICA) riboside. Another approach to increase endogenous Ado concentration, mainly in the ischemic heart, is the inhibition of cell membrane transport of Ado. Ado concentration in the ischemic heart could also be increased by the application of inhibitors of Ado deaminase such as erythroid-9(2-hydroxy-3-nonyl) adenine (EHNA). This approach provides significant improvement in myocardial function and metabolism after ischemia.

The combined inhibition of the Ado kinase and Ado deaminase activities allows increased Ado production not only under ischemic conditions but also in the normoxic heart. Based on this approach, we recently developed a procedure that allows sustained increase in endogenous Ado production in cultured cardiomyocytes and endothelial cells. We identified initially that the application of Ado metabolism inhibitors increases Ado production, although at the expense of adenine nucleotide pool depletion in the cells. However, we further demonstrated that the combined application of Ado metabolism inhibitors with nucleotide precursors increases the production of Ado and simultaneously protects or even elevates the adenine nucleotide pool. An important part of this study was optimization of the concentration and type of substrates and inhibitors to maximize Ado production and to minimize unwanted effects. A major advantage of our approach is that it offers the potential to increase Ado production not only during the ischemic or reperfusion phase but also under normoxic conditions, which may considerably extend the time window of the beneficial effects of Ado.

The objective of the present study was to establish whether sustained increase in endogenous Ado production through the inhibition of Ado kinase and Ado deaminase, combined with adenine and ribose, is protective in a protocol mimicking preservation for cardiac transplantation followed by heterotopic implantation and up to 24 hours of reperfusion.

## Methods

### Animals

All animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication No. 85-23, revised 1985). This project was approved by the Institutional Ethics Committee on Research Involving Animals at Harefield Hospital. Male Sprague-Dawley rats (275 to 300 g) were used as both the donors and the recipients and in all other experiments described here.

### Physiological and Metabolic Effects of Ado Metabolism Inhibitors/Nucleotide Substrates

To study metabolic and physiological effects of the administration of Ado inhibitors/nucleotide substrates, rats were anesthetized with diethyl ether and intraperitoneally injected with a solution containing: 0.56 mmol/L 5'-aminoadenosine (Ado kinase inhibitor [AA]), 0.28 mmol/L erythroid-9(2-hydroxy-3-nonyl) adenine (EHNA), 280 mmol/L ribose (R), and 5.6 mmol/L adenine (A) at a dose of 15 mL/kg. Blood samples were collected from the femoral vein before injection and 5, 15, and 60 minutes and 6, 12, and 24 hours after the injection. Samples were immediately (within 3 seconds) frozen in liquid nitrogen and stored until extracted with perchloric acid. In another set of experiments, rats were anesthetized with 50 mg/kg pentobarbital IP, and a saline-filled cannula connected to a pressure transducer was introduced into the femoral artery. After stabilization of the blood pressure recording, the Ado inhibitor/nucleotide substrate mixture was injected at the dose stated, and changes in blood pressure were monitored for 60 minutes.

### Effect of Ado Metabolic Inhibitors/Nucleotide Substrates on Ischemia-Reperfusion Injury in Transplanted Hearts

To study the effect of Ado metabolism inhibitors/nucleotide substrates on ischemia-reperfusion injury in a protocol mimicking preservation for cardiac transplantation, heterotopic transplant experiments were performed as presented in Figure 1. Donor rats were anesthetized with diethyl ether and heparinized (200 IU/kg IV). Then, the abdominal and chest cavities were opened, and hearts were collected after the in situ infusion of 30 mL St Thomas’ Hospital No. 1 cardioplegic solution through the abdominal aorta. After the ligation of cardiac veins, the hearts were collected and stored in cardioplegic fluid for 4 hours at 4°C. Then, hearts were heterotopically transplanted into the abdomen of recipient rats. The transplantation procedure generally followed that described previously Briefly, the abdominal cavity of the recipient rat, which was anesthetized with pentobarbital (50 mg/kg IP), was opened, and the aorta of the donor heart was anastomosed to the side of the recipient’s abdominal aorta. The pulmonary artery of the donor heart
was anastomosed to the side of the inferior vena cava. The transplantation procedure was completed within 20 minutes. In the treated groups, the AA/EHNA/A/R solution (described earlier) was administered at 15 mL/kg into the peritoneal cavity at the time the abdomen was closed. In the control group, 0.9% saline was administered at the same volume. After transplantation, hearts were collected either after 1 hour or the rats were allowed to recover and were maintained for 24 hours. At that time, the rats were anesthetized, and the transplanted hearts were collected for functional and metabolic assessment as described later.

Functional Assessment in Ex Vivo Perfusion System

Rats were anesthetized with diethyl ether and heparinized (200 IU) via the femoral vein. The abdominal cavity was opened, and the hearts were rapidly excised, placed in ice-cold perfusion buffer, and immediately attached to a Langendorff perfusion system. The hearts were then perfused with filtered (0.45-µm pore size) Krebs-Henseleit buffer solution at a constant pressure of 85 mm Hg at 37°C as described previously. The buffer solution contained (in mmol/L) NaCl 118, KCl 4.7, MgSO4 1.2, KH2PO4 1.2, NaHCO3 24, glucose 11, and CaCl2 1.4 and was continuously gassed with 95% O2/5% CO2. Assessment of mechanical function was made using a balloon catheter inserted into the left ventricle and connected a pressure transducer linked to a PC-based data acquisition and processing system (AcqKnowledge; Biopac Systems Inc). The balloon was inflated with incremental volumes of water from 0 to 250 µL (50 µL each step). Pressure recordings were used to construct left ventricular systolic pressure (LVSP) and left ventricular end-diastolic pressure (LVEDP) volume relations. A difference between LVSP and LVEDP was used to calculate left ventricular developed pressure (LVEDP) volume relations. Other information, such as heart rate, maximum value of +dP/dt, and minimum value of −dP/dt, were derived from recorded data. Coronary flow was continuously recorded with an electromagnetic flowmeter (Nihon-Kohden).

Myeloperoxidase Assay and Measurement of Cardiac Nucleotide Concentration

Activity of the neutrophil marker enzyme myeloperoxidase was measured in homogenates of hearts freeze clamped after functional assessment at the end of Langendorff perfusion. The spectrophotometric assay procedure followed that described previously. Concentrations of nucleotides and creatine metabolites were measured in hearts using reversed-phase HPLC as described in detail previously, except that freeze-clamped hearts were freeze dried and extracted with 0.4 mol/L perchloric acid. After neutralization, samples were analyzed with the Agilent 1100 HPLC system.

Statistical Analysis

All values are presented as mean±SEM. Statistical analysis of the differences in systolic and diastolic pressure-volume relations was made with 2-way ANOVA, followed by the Student-Newman-Keuls test. Changes in myeloperoxidase activity, metabolite concentration, developed pressure, +dP/dt, and −dP/dt were analyzed using 1-way ANOVA, followed by the Student-Newman-Keuls test. If data distribution was not normal or variance was not equal, ANOVA for ranks was used. Differences were considered significant at P<0.05.

Results

Ado Concentration and Other Metabolic and Physiological Changes After Ado Metabolic Inhibitor/Nucleotide Substrate Administration

The effect of the in vivo administration of Ado metabolism inhibitors/nucleotide substrates was studied in anesthetized rats after the intraperitoneal injection of AA/EHNA/A/R solution. Blood Ado concentration increased 5 minutes after the injection, peaked after 15 minutes (8± initial concentration), and remained elevated (3± initial concentration) at 6 hours after the injection (Figure 2A). The concentration of hypoxanthine and uric acid increased shortly after the injection of AA/EHNA/A/R solution, and xanthine increased at the later stages (Figure 2B). The concentrations of all purines returned to normal levels 24 hours after the injection of Ado metabolism inhibitors/nucleotide substrates. No significant changes in blood purine concentration were observed after the administration of saline (data not shown). The initial blood concentration of creatinine was 430.1±36.3 µmol/L, and it remained constant both after the injection of Ado metabolism inhibitors/nucleotide substrates and after the injection of saline. Mean arterial blood pressure measured in the femoral artery of the anesthetized rats was 97±6 mm Hg. Maximum decrease in blood pressure occurred between 5 to 20 minutes after the injection of Ado metabolism inhibitors/nucleotide substrates and then gradually recovered. The degree of drop in blood pressure ranged from 8 to 17 mm Hg in different experiments. Slight decreases in blood pressure were also observed after the administration of saline.

Effect of Ado Metabolic Inhibitors/Nucleotide Substrates on Reperfusion Injury in Transplanted Hearts

The administration of Ado metabolism inhibitors/nucleotide substrates resulted in a marked improvement in all aspects of
cardiac function after 24 hours of reperfusion, whereas no differences were observed between treated and control animals after 1 hour of reperfusion (Figure 3). As may be seen, both systolic (LVDP_{max}, +dP/dt) and diastolic (end-diastolic pressure-volume relation, −dP/dt) functions of transplanted hearts were improved in treated hearts after 24 hours' reperfusion. This improvement in treated hearts versus control hearts after 24 hours of reperfusion resulted from further deterioration in cardiac function in control hearts between 1 and 24 hours of reperfusion (Figure 3), which was prevented by the administration of Ado metabolism inhibitors/nucleotide substrates. Functional differences in hearts reperfused for 24 hours were accompanied by accelerated recovery of ATP concentration (35% increase versus control) in treated hearts (Figure 4). No major changes in the content of other measured metabolites were observed, with the exception of a higher creatine concentration in the treated group compared with the control group. Also, a lower concentration of 5'-adenosine-diphosphoribose (NADH catabolite [ADPR]) and a higher concentration of NADP were observed in transplanted hearts compared with nontransplanted hearts. As may be seen from Figure 5, the administration of Ado metabolism inhibitors/nucleotide substrates resulted in marked attenuation of neutrophil infiltration in hearts reperfused for 24 hours, as indicated by reduced activity of myeloperoxidase in treated hearts. Interestingly, very little increase in myeloperoxidase was observed after 1 hour of reperfusion. No differences were observed between treated and control hearts, and in fact, only a small increase was observed compared with nontransplanted hearts. ATP concentration was similar in treated and control hearts after 1 hour of reperfusion. Heart rate was not significantly different between treated and control groups in both the 1-hour reperfusion and the 24-hour reperfusion groups. However, heart rate was decreased by ≈20% compared with nontransplanted hearts.
substrate solution increased Ado concentration for continuous Ado administration within that time would be necessary. 

Control 24-hour reperfusion groups.

Inhibitors of Ado metabolism/nucleotide substrates administered heterotopic transplantation, and reperfusion for 1 or 24 hours. 

Figure 5. Activity of neutrophil-specific enzyme myeloperoxidase in rat hearts subjected to 4 hours of cardioplegic arrest, heterotopic transplantation, and reperfusion for 1 or 24 hours. 

The present study demonstrated that the administration of Ado metabolism inhibitors together with precursors of nucleotide synthesis caused prolonged elevation in Ado concentration and exerted potent protective effect in hearts subjected to conditions mimicking preservation for transplantation. In addition to improvement in all aspects of cardiac mechanical function and an elevated ATP level, a marked 4-fold reduction in neutrophil infiltration was observed. This study underlined the importance of reperfusion injury and postischemic inflammation in cardiac transplantation. There was substantial deterioration in mechanical function between 1 hour and 24 hours of reperfusion in control hearts, which was almost completely prevented by our proposed intervention. 

The results presented here highlight that our Ado metabolism inhibitor/nucleotide substrate combination is well tolerated in vivo. Thus, a similar procedure could be a valuable treatment in clinical transplantation and possibly in other situations associated with cardiac ischemia-reperfusion or inflammatory reaction.

Functional improvement is the most important end point of any cardioprotective strategy. In the present experiments, both systolic and diastolic functions were considerably improved in hearts treated with Ado metabolism inhibitors/nucleotide substrates. This is in line with earlier observations related to the protective effect of Ado supply during reperfusion under conditions mimicking long-term preservation for transplantation. However, the administration of Ado metabolism inhibitors/nucleotide substrates is superior in several aspects to direct Ado infusion. A single dose of inhibitor/substrate solution increased Ado concentration for >6 hours. To achieve a similar effect through direct infusion, a continuous Ado administration within that time would be necessary. Furthermore, the possibility of repeated administration of Ado metabolism inhibitors/nucleotide substrates offers the potential to regulate Ado production for days and weeks, which is impossible to achieve through direct Ado infusion even when using osmotic pumps. Another advantage of endogenously produced Ado is better compartmentation compared with direct Ado infusion. Due to rapid Ado metabolism, only a small proportion of intravenously infused Ado will reach cardiac cells. Intracoronary infusion may increase cardiac concentration but requires catheterization with all its risks, costs, and complications.

An increase in Ado concentration after the administration of Ado metabolism inhibitors/nucleotide substrates is directly related to the rate of Ado cycle (Ado phosphorylation to AMP/dephosphorylation of AMP to Ado) in a particular cell type. Although the Ado cycle operates in many cells, including erythrocytes, hepatocytes, neutrophils, and lymphocytes, the rate in endothelial cells seems to be the highest with the rate in cardiomyocytes only slightly lower. Thus, it is reasonable to assume that Ado will accumulate preferentially in the vicinity of endothelium and cardiomyocytes, which is optimal to exert cardioprotective effects. One technical concern is the fact that intraperitoneal administration was used in our study, because it is more practical during heterotopic transplantation procedure. This may not be directly compatible with a potential clinical application of the Ado inhibitor/nucleotide substrate mixture. However, only adjustments in dose and duration of infusion would be necessary to achieve similar metabolic effects after intravascular administration.

Due to the broad spectrum of Ado activities in various organs and cell types as well as the involvement of receptor-mediated and metabolic effects, it is difficult to identify the primary mechanism of the effect observed in the present study. However, two observations made in our study, elevated ATP and marked reduction in neutrophil infiltration in hearts treated with Ado metabolism inhibitors/nucleotide substrates, allows some speculation on the mechanism. High ATP on its own may exert a beneficial effect on cardiac function, including improvement in the diastolic properties of the heart. Although a direct relation between contractility and ATP concentration has been questioned, others still consider this possibility. Little is known, in particular, about the long-term effects of a decreased nucleotide pool on contractility and nucleotide-dependent regulatory mechanisms in the cardiac cells. It is important to note that in accordance with our isolated cell experiments, the supply of adenine and ribose allowed not only prevention of nucleotide depletion due to Ado kinase inhibition but also markedly accelerated recovery of the adenine nucleotide pool. On the other hand, a marked reduction in neutrophil infiltration, indicating attenuation of postischemic inflammation, demonstrated in our experiments may highlight another mechanism, which facilitates functional improvement. A significant role of this mechanism has been identified in earlier studies, and the degree of the difference observed in our experiments underlies the importance of this possibility. It is difficult to identify the exact mechanism of this complete block of neutrophil infiltration, but several possibilities could be considered. A direct Ado effect on neutrophil adhesion has long been
recognized. However, reduction in the endothelial expression of adhesion molecules could be an alternative explanation. This latter possibility is supported by the observation that the peak Ado concentration occurred 15 minutes after Ado metabolism inhibitor/nucleotide substrate administration, whereas a protective effect was observed 24 hours after treatment, which is consistent with the time necessary for a change in protein expression. Regardless of the mechanism, the observation that Ado metabolism inhibitor/nucleotide substrate administration is capable of completely abolishing neutrophil infiltration in the postischemic heart is important, because this procedure may be more practical than and equally effective as more specific interventions.

Several other options not addressed in the present study have to be taken into consideration. The time course of the protective effect suggests that attenuation of the apoptotic mechanism, which is now considered an important component of reperfusion injury, could play a significant role in our experiments. An increase in Ado production and attenuation of postischemic inflammation may protect, in particular, endothelium and its function. Furthermore, the effect of Ado on platelet aggregation, glucose metabolism, and free radical production may have played a role. It is likely that the effect of Ado inhibitors/nucleoside substrates on protection from reperfusion injury in our study resulted from a combination of the different effects of Ado. A direct protective effect of other constituents of the solution administered, ribose or adenine, as well as other metabolic changes, such as elevated uric acid (potent antioxidant) concentration, cannot be ruled out. However, a major objective of the present study was to establish in vivo effect of inhibitor/substrate combination optimized in our cultured cell experiments and based on extensive existing knowledge of Ado metabolism and effects.

The beneficial effects of Ado metabolism inhibitor/nucleotide substrate combination studied here are not restricted to reperfusion injury. In our other experiments, we have shown extended survival and improvement of graft function in allogenic (F344 to Lewis) rat heart transplants after this treatment, which indicated attenuation of acute rejection mechanisms. Ado shows well-characterized immunosuppressive properties such as inhibition of blastic transformation of lymphocytes and inhibition of its cytoxic effects. It was recently found that Ado may attenuate humoral immune mechanisms by induction of changes in endothelium. This further suggests that the administration of Ado metabolism inhibitors/nucleotide substrates could be particularly useful during clinical cardiac transplantation. These studies also indicated that the administration of our inhibitor/substrate combination is well tolerated not only after a single injection but also after days or weeks of continued treatment.

The administration of Ado metabolism inhibitors/nucleotide substrates as a single or prolonged treatment did not cause any major adverse effects, although several possibilities have to be taken into consideration. Enhanced Ado production may result in impaired renal function due to Ado-mediated renal vasoconstriction, and elevated adenine may result in kidney stone formation. Furthermore, an increased Ado concentration may affect blood pressure and heart rate. Our results indicated no changes in creatinine level not only in the present study but also in other experiments with prolonged treatments with Ado metabolism inhibitors/nucleotide substrates, indicating adequate maintenance of renal function. Although some decrease in blood pressure has been observed after the administration of Ado metabolism inhibitors/nucleotide substrates, it was relatively small. This mild effect on blood pressure at such a high blood Ado concentration is surprising but may indicate effective compensation by other mechanisms of vascular tone regulation. Relatively mild effects on the heart rate were observed. Although the administration of Ado metabolism inhibitors/nucleotide substrates induced bradycardia in both transplanted and own-recipient hearts, the rate in the latter never decreased below 250 bpm. No long-term effects on heart rhythm in transplanted hearts were noted, because the rate under standardized conditions during Langendorff perfusion did not differ between the groups.

An important question is whether the work presented here is applicable to humans despite the number of differences in nucleotide metabolism and mechanisms or ischemia-reperfusion injury and rejection. Our studies with human endothelial cells isolated from capillaries of the heart indicated a generally similar increase in Ado production after the inhibition of Ado kinase and Ado deaminase. There were considerable differences in adenine incorporation, but the capacity of this pathway in human endothelium was sufficient to maintain the adenine nucleotide pool. Caution may be required with the selection of Ado deaminase inhibitor because EHNA was less effective in human endothelium.

The present study highlighted that inhibition of Ado deaminase and Ado kinase combined with precursors of nucleotide synthesis adenine and ribose at the time of reperfusion causes an increase in endogenous Ado production and results in sustained improvement of mechanical function, elevated ATP concentration, and marked attenuation of inflammation. This intervention could be an effective treatment that alleviates several diverse clinical problems during clinical cardiac transplantation.

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References
7. Galinanes M, Hearse DJ. Exogenous adenosine accelerates recovery of cardiac function and improves coronary flow after long-term hypothermic...
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