Augmentation of Endogenous Adenosine Attenuates Myocardial ‘Stunning’ Independently of Coronary Flow or Hemodynamic Effects

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Background. Mounting evidence suggests a protective effect of exogenous adenosine in myocardial ischemia and reperfusion. We tested the hypothesis that augmentation of endogenous adenosine levels, achieved by inhibiting adenosine catabolism and washout, is beneficial in postischemic myocardial dysfunction (“stunning”).

Methods and Results. In phase I of the study, open-chest dogs undergoing a 15-minute coronary artery occlusion and 4 hours of reperfusion received an intracoronary infusion of either saline (controls, n=23) or 6-(4-nitrobenzyl)-mercapto: purine ribonucleoside (NBMPR, a selective nucleoside transport inhibitor) combined with erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA, a potent adenosine deaminase inhibitor) (EHNA+NBMPR, n=15) starting 15 minutes before coronary occlusion and ending 15 minutes after the initiation of reflow. Regional myocardial function (assessed as systolic wall thickening) was similar in control and treated groups at baseline and during ischemia. After reperfusion, however, the dogs treated with EHNA+NBMPR exhibited a significant improvement in the recovery of function, which was evident as early as 30 minutes after restoration of flow and was sustained throughout the rest of the reperfusion phase. The enhanced recovery effected by EHNA+NBMPR could not be attributed to nonspecific factors such as differences in collateral flow during occlusion, coronary flow after reperfusion, arterial pressure, heart rate, or other hemodynamic variables. In phase II of the study, the myocardial content of adenine nucleotides and nucleosides was measured by high performance liquid chromatography in myocardial biopsies obtained serially from open-chest dogs undergoing the same protocol used in phase I. There were no significant differences between control (n=8) and treated (n=9) dogs with respect to myocardial levels of adenosine triphosphate (ATP) at 30 and 60 minutes after reperfusion, indicating that the beneficial effects of EHNA+NBMPR cannot be ascribed to repletion of ATP stores. Compared with controls, dogs treated with EHNA+NBMPR exhibited a much larger increase in myocardial adenosine (6.07±1.47 vs 1.03±0.16 nmol/mg protein; P<.05) and a much smaller increase in inosine (0.52±0.27 vs 3.04±0.54 nmol/mg protein, P<.05) at the end of ischemia, such that the inosine-to-adenosine ratio noted in controls was completely reversed (≈6:1 vs ≈1:6, respectively). In the treated group, adenosine levels remained markedly increased compared with controls up to 1 hour after reperfusion.

Conclusions. This study demonstrates that (1) administration of an adenosine deaminase inhibitor plus a nucleoside transport blocker is remarkably effective in augmenting myocardial adenosine levels during regional ischemia and subsequent reperfusion in vivo, (2) this augmentation of adenosine results in a significant and sustained attenuation of myocardial stunning, and (3) the attenuation of stunning is not due to ATP repletion or to nonspecific actions on hemodynamic variables or coronary flow. These findings suggest that endogenous adenosine production during ischemia serves as an important pathophysiological mechanism that protects against myocardial stunning. The results also suggest that augmentation of endogenous adenosine (without exogenous adenosine administration) represents an effective therapeutic approach to the alleviation of reversible postischemic dysfunction. (Circulation. 1993;88[part 1]:2359-2369.)

KEY WORDS • stunned myocardium • adenosine • nucleotides • ischemia

Postischemic myocardial dysfunction, or myocardial “stunning,”1 is the protracted depression of contractility that is observed after a reversible ischemic insult.2-3 In view of the mounting evidence that myocardial stunning occurs clinically and may be a significant cause of morbidity,4 intense research has focused on the pathogenesis of this phenomenon and on the development of effective therapies. Recently, there has been considerable interest in the possible cardioprotective effects of adenosine during myocardial ischemia and reperfusion.5-7 Adenosine possesses several

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properties that could be beneficial in this setting, including replenishment of ATP stores, coronary vasodilation, stimulation of glycolysis, inhibition of leukocyte function, inhibition of calcium transport, and inhibition of lipolysis and attendant free radical generation.\(^a\)\(^b\)\(^c\)\(^d\) Indeed, adenosine has been reported to decrease infarct size and improve mechanical function after a prolonged (90 to 120 minutes) coronary occlusion in closed-chest dogs and to mitigate contractile dysfunction after brief (<20 minutes) ischemic periods followed by reflow in isolated rat hearts\(^e\)\(^f\) and after prolonged global ischemia in models of cardiopulmonary bypass in dogs.\(^g\)\(^h\)\(^i\) Adenosine A\(_1\)-receptor agonists have been found to be beneficial in isolated rat hearts subjected to global ischemia,\(^j\)\(^k\)\(^l\) suggesting that adenosine enhances cardiac tolerance to ischemia via activation of A\(_1\)-receptors. There is also indirect evidence for a salutary effect of adenosine in experiments where its production was stimulated by \(\alpha\)-adrenergic receptor agonists.\(^m\)\(^n\)

These experiments\(^p\)\(^q\)\(^r\)\(^s\)\(^t\)\(^u\) have demonstrated the cardio-protective actions of exogenous adenosine. Administration of adenosine, however, has disadvantages because the plasma half-life of this nucleoside is extremely short due to its rapid removal from the bloodstream and its cellular uptake and metabolism via adenosine kinase and adenosine deaminase.\(^v\) This mandates infusion of pharmacological doses of adenosine, which may lead to a host of undesirable side effects, including arterial hypotension, reflex tachycardia, and renal vasoconstriction. In addition, adenosine infusion may produce a high urate load on the kidneys and may enhance the degradation of this nucleoside through the xanthine oxidase pathway, thereby potentially promoting oxradical production. A different approach is to increase endogenous adenosine levels by inhibiting its degradation (eg, with adenosine deaminase inhibitors) or its washout (eg, with nucleoside transport inhibitors). Numerous studies\(^w\)\(^x\)\(^y\)\(^z\)\(^{aa}\) have suggested that these manipulations are beneficial in models of myocardial ischemia and reperfusion, but most of these studies did not specifically examine myocardial stunning. Furthermore, some of these investigations were performed in isolated heart models\(^bb\)\(^bc\)\(^bd\)\(^be\) and some\(^bf\)\(^bg\) were performed in guinea pigs, a species known to have a unique nucleoside metabolism.\(^bh\)\(^bi\) A number of studies\(^bj\)\(^bk\)\(^bl\)\(^bm\)\(^bn\) used substances (like miofazine) that are known to have calcium channel blocking properties in addition to nucleoside transport inhibitor properties.\(^bo\)\(^bp\) Several studies\(^bq\)\(^br\)\(^bs\)\(^bt\) demonstrated protective effects of adenosine deaminase and nucleoside transport inhibitors in dogs subjected to global myocardial ischemia and cardiopulmonary bypass; however, because of the numerous differences between global and regional ischemia, the applicability of these results to the setting of regional myocardial stunning after coronary occlusion is uncertain.

The goals of this study were to determine (1) whether regional myocardial stunning can be attenuated by manipulations that augment endogenous adenosine levels during ischemia and reperfusion and (2) if so, whether such a salutary effect is independent of hemodynamic factors. To this end, we investigated the effect of the combination of \(\text{erythro-9-(2-hydroxy-3-nonyl)adenine}\) (EHNA), a potent adenosine deaminase inhibi-

tor, and 6-(4-nitrobenzyl)-mercaptopurine ribonucleoside (NBMPR), a specific nucleoside transport inhibitor, in a canine model of 15 minutes of ischemia followed by 4 hours of reflow.\(^ct\)\(^cu\)\(^cv\)\(^cw\)\(^cx\) Particular care was taken to ensure that any beneficial effect observed with EHNA+NBMPR could not be ascribed to nonspecific actions of these agents on systemic hemodynamics, collateral flow during coronary occlusion, or coronary flow after reperfusion.

### Methods

A total of 106 dogs was used (including the pilot studies). The study consisted of two phases. The goal of phase I was to investigate the effects of EHNA+NBMPR on the recovery of contractile function after reperfusion. The goal of phase II was to determine the effects of EHNA+NBMPR on the tissue levels of adenosine nucleotides and nucleosides. All experiments were performed in accordance with the guidelines of the Committee on Animals of Baylor College of Medicine and with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 80-23).

#### Phase I

**Experimental preparation.** The experimental preparation has been described in detail previously.\(^cv\)\(^cw\) Briefly, pentobarbital-anesthetized dogs were instrumented with a snare around the mid left anterior descending artery (LAD), a Doppler flow velocity probe around the LAD adjacent to the snare, a 6F Millar pressure transducer (Millar Instruments, Houston, Tex) in the left ventricle through an apical stab wound, Doppler ultrasonic wall thickening probes in the ischemic and nonischemic zones, and left atrial and aortic catheters. In addition, a 27-gauge needle connected to a lymphangiographic catheter was placed into the proximal LAD for infusion of EHNA+NBMPR solution or saline. To prevent clotting, heparin was given immediately after insertion of the needle (3000 U IV) and continuously thereafter (500 U/h). Peripheral venous access was established through the external jugular vein and one of the limb veins.

Particular care was taken to ensure that fundamental physiological parameters were within normal limits for the duration of the protocol. Only dogs with hematocrit >30%, \(\text{Pa}_2 > 60 \text{ mm Hg}\), and \(\text{pH} > 7.36\) were included. Supplements of KCl were given intravenously to keep plasma potassium concentration between 4.0 and 5.0 mEq/L.\(^cx\) The rate of fluid replacement was standardized at approximately 80 mL/min.\(^cz\) Esophageal temperature was kept constant at \(=38^\circ\text{C}\) throughout the study by adjusting a heating blanket. Arterial blood gases were kept in the normal range by adjusting the ventilatory parameters. The importance of controlling body temperature has been stressed previously.\(^{da}\) We have shown that if no measure is taken to maintain normothermia, body temperature declines progressively, which results in artifactual enhancement of regional function.\(^{db}\) This is the most likely reason for the difference in the degree of stunning observed between the control (normothermic) dogs in the present study and the control dogs of previous studies (for example, see Reference 33) in which temperature was not controlled.

**EHNA+NBMPR solution.** A solution containing 425 \(\mu\)mol/L EHNA and 87 \(\mu\)mol/L NBMPR was prepared.
as follows. Twenty milligrams of EHNA (Sigma Chemical Co, St Louis, Mo; purity, 99%) was dissolved in 147 mL of normal saline; 5.5 mg of NBMPR (Aldrich Chemical Co; purity, 99.8%) was dissolved separately in 0.28 mL of dimethylsulfoxide, then added slowly to the EHNA solution with gentle stirring to avoid the formation of a suspension. The solution obtained was protected from light, and the pH was adjusted to 7.40.

Pilot studies. A series of pilot studies was conducted in 12 dogs to find an appropriate dosage regimen of EHNA+NBMPR. These two agents can produce arterial hypotension and coronary vasodilatation; since both of these effects in themselves can increase systolic shortening in the postischemic myocardium, their presence would have confounded the interpretation of our study. Therefore, our goal was to identify a dosage that would provide effective drug tissue levels and, at the same time, either eliminate any effect on systolic arterial pressure and coronary flow or restrict the changes in these parameters to the duration of the infusion so that they would not confound the later phases of the experiment. Seven different treatment protocols were evaluated. Initially, EHNA+NBMPR were infused intravenously, but we found that this modality of administration was associated with arterial hypotension and with an increase in LAD flow that persisted until 4 hours after reperfusion. We then evaluated intracoronary administration, and we decreased the dosage of EHNA+NBMPR until we identified a protocol that did not cause persistent hypotension or persistent hyperemia.

Protocol for administration of EHNA+NBMPR. Finally, the following protocol was adopted. An intracoronary infusion of EHNA+NBMPR solution (425 \(\mu\)mol/L and 87 \(\mu\)mol/L, respectively) was given at a rate corresponding to 10% of baseline LAD flow; this resulted in calculated coronary arterial plasma concentrations of 39 \(\mu\)mol/L EHNA and 8 \(\mu\)mol/L NBMPR. The infusion was normalized to coronary flow in an attempt to achieve similar coronary arterial concentrations of drugs in all dogs. The infusion was started 15 minutes before coronary occlusion and continued until 15 minutes after reperfusion. During the first 13.5 minutes of coronary occlusion, the rate of infusion was decreased to 0.6% of baseline flow to take into account the low level of collateral perfusion present in the open-chest dog. The baseline rate of infusion was resumed 90 seconds before reperfusion and maintained throughout the first 15 minutes of reperfusion. The reason for increasing the rate of infusion 90 seconds before reflow was to ensure that high local concentrations of EHNA+NBMPR were present in the LAD bed at the onset of reperfusion. Control dogs received vehicle at the same rate and with the same timing as the EHNA+NBMPR solution. The pilot studies mentioned above indicate that this treatment protocol enabled us to administer the highest doses of EHNA and NBMPR that can be given without producing systemic hemodynamic effects or prolonged coronary hyperemia.

Experimental protocol. All dogs underwent a 15-minute LAD occlusion followed by 4 hours of reperfusion. Regional myocardial blood flow was determined 5 minutes after coronary occlusion by injecting radioactive microspheres. The technique for microsphere injection, processing of tissue, and calculation of flow has been described in detail previously. At the conclusion of the study, the size of the occluded bed was determined by a previously described postmortem perfusion technique in which the previously occluded LAD was perfused with 1% triphenyltetrazolium chloride (TTC) to verify absence of infarction while the proximal LAD and circumflex arteries were simultaneously perfused with 0.5% Monastral blue at equal physiological pressures (100 mm Hg). The occluded bed weight was expressed as a percentage of left ventricular (LV) weight. Regional myocardial function was assessed as systolic wall thickening using an epicardial Doppler probe, as previously described. The beginning and end of systole were determined from the onset of the rapid upstroke of the LV pressure tracing and the peak negative LV dP/dt, respectively. Percent systolic thickening fraction was calculated as the ratio of systolic thickening to end-diastolic thickness, multiplied by 100.

Phase II
A series of separate experiments was conducted to determine the effect of EHNA+NBMPR on the content of adenine nucleotides and nucleosides in the myocardium. These measurements could not be performed in phase I because the trauma of repeated biopsies would have interfered with systolic wall thickening. Briefly, pentobarbital-anesthetized open-chest dogs, prepared as described above, underwent a 15-minute LAD occlusion followed by 1 hour of reperfusion. Treated dogs received EHNA and NBMPR exactly as described in phase I, whereas controls received equivalent volumes of vehicle.

Assessment of adenine nucleotide pool metabolism. Serial myocardial biopsies from the ischemic and nonischemic regions were obtained with a Tru-Cut needle (Travenol Laboratories, Deerfield, Ill) at baseline (before treatment), 5 minutes before occlusion (during treatment), at the end of the 15-minute ischemic period, and 30 and 60 minutes after reperfusion. Biopsies were immediately frozen in liquid nitrogen. Each biopsy specimen was extracted with 12% trichloroacetic acid (4°C) for 30 minutes with frequent homogenization. The soluble acid extract was separated from denatured protein by centrifugation and neutralized with a tri-n-octylamine-freon mixture (1:3 vol/vol). The protein in the pellet was determined by the method of Lowry et al. The neutralized extracts were stored at −70°C. Myocardial ATP, ADP, AMP, adenosine, inosine, hypoxanthine, xanthine, and NAD+ were determined with high performance liquid chromatography as previously described. The results were expressed as nanomoles per milligram of protein.

Statistical Analysis
All values are reported as mean±SEM. Comparisons were performed with a two-way ANOVA for repeated measures; when an overall significant difference was detected, pairs of means were compared using Tukey's post hoc tests. The correlation between thickening fraction at 4 hours of reperfusion and transmural collateral blood flow was examined using linear regression analysis, and comparisons between the two groups were made using an ANCOVA in which collateral flow was the covariate. A P value <.05 was considered statistically significant.
### Table 1. Reasons for Excluding Dogs From Phase I of the Study

<table>
<thead>
<tr>
<th>Reason</th>
<th>EHNA+NBMPR Group</th>
<th>Control Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abnormal baseline hemodynamics</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>(heart rate &gt;200 bpm or hypoxemia (Po2 &lt;50 mm Hg))</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Technical problems during instrumentation</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Technical problems with intracoronary needle (clotting or bleeding)</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>EHNA+NBMPR infusion resulted in marked systemic effects (hypotension)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Ventricular fibrillation upon reperfusion</td>
<td>3</td>
<td>6</td>
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<tr>
<td>Myocardial infarction (subendocardial)</td>
<td>2</td>
<td></td>
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<tr>
<td>High collateral flow to ischemic zone (&gt;30% of NZF)</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>Total number of dogs excluded</td>
<td>19</td>
<td>20</td>
</tr>
<tr>
<td>Total number of dogs anesthetized</td>
<td>34</td>
<td>43</td>
</tr>
<tr>
<td>Total number of dogs included in study</td>
<td>15</td>
<td>23</td>
</tr>
<tr>
<td>Percentage of total</td>
<td>44%</td>
<td>53%</td>
</tr>
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</table>

EHNA indicates erythro-9-(hydroxy-3-nonyl)adenine; NBMPR, 6-(4-nitrobenzyl)-mercaptopurine; bpm, beats per minute; and NZF, nonischemic zone flow.

### Results

#### Phase I

**Exclusions.** Of the 77 dogs initially anesthetized in phase I, 39 (51%) were excluded for the reasons specified in Table 1. Thus, analysis of data was carried out in 23 control and 15 treated dogs. Although our previous experience and numerous other studies have shown absence of irreversible tissue damage after a 15-minute coronary occlusion in dogs, two animals in our treated group exhibited small subendocardial infarctions on the postmortem TTC staining. The locations of these infarcts suggested clotting at the site of the intracoronary needle as the cause of necrosis.

**Arterial blood gases, hematocrit, and body temperature.** Arterial Po2, pH, hematocrit, and esophageal temperature were within physiological limits in the two groups throughout the experimental protocol (data not shown). The total supplemental doses of pentobarbital given to maintain anesthesia were similar: 7.0±0.6 mg/kg in control and 9.7±0.5 mg/kg in treated dogs.

**Hemodynamic variables.** The administration of EHNA+NBMPR had no significant effect on heart rate, arterial pressure, rate-pressure product, and positive LV dP/dt during the infusion of the drugs (Table 2). Arterial pressure decreased slightly in the treated group at the end of the reperfusion phase (4 hours), but this late effect cannot account for the enhanced recovery observed at earlier time points (see below). Thus, our dosage of EHNA+NBMPR did not produce any systemic hemodynamic changes during most of the experiment.

Heart rate, arterial pressure, and the rate-pressure product were slightly higher in treated compared with control dogs at baseline (before treatment) and at several time points thereafter (Table 2). Because of the increased afterload and myocardial oxygen demands, these differences, if anything, would be expected to prevent a beneficial effect of EHNA+NBMPR from becoming manifest.

Coronary flow in the LAD increased by 63% (P<.05) with the infusion of EHNA+NBMPR (Table 2). After reperfusion, LAD flow achieved higher levels than before occlusion and remained at these levels throughout the infusion of EHNA+NBMPR and even 15 minutes after its termination (30 minutes after reperfusion). However, LAD flow returned to values not significantly different from baseline by 1 hour of reperfusion (Table 2). There were no significant differences in LAD flow between control and treated dogs at 1, 2, 3, or 4 hours of reflow, indicating that our dosage of EHNA+NBMPR did not produce any hyperemia beyond the first 30 minutes of reperfusion.

**Occluded bed size and regional myocardial blood flow.** As shown in Table 3, the size of the occluded vascular bed was similar in the EHNA+NBMPR and in the control groups. The measurements of regional myocardial blood flow are also summarized in Table 3. During coronary occlusion, blood flow to the nonischemic region was significantly higher in the EHNA+NBMPR group than in the control group (P<.01), a difference probably caused by recirculation of the drugs. Collateral blood flow to the ischemic zone, however, was similar in the EHNA+NBMPR group and in the control group: 0.14±0.03 vs 0.12±0.02 mL·min⁻¹·g⁻¹, respectively.

**Regional myocardial function.** In both control and treated dogs, systolic thickening fraction in the nonischemic (control) region increased transiently during occlusion and then exhibited the progressive deterioration that is usually seen in open-chest animals (Fig 1). There were no differences in the measurements of thickening fraction in the nonischemic region between the two groups at any time point during the protocol (Fig 1). Baseline systolic thickening fraction in the region to be rendered ischemic (Fig 2) was 25.5±1.2% and 26.3±1.3% in control and EHNA+NBMPR-treated dogs, respectively (P=NS). The infusion of EHNA+NBMPR did not produce any appreciable change in thickening fraction (preocclusion measurement: 98.3±2.1% of baseline). During coronary occlusion, the extent of paradoxical thinning was also similar in the two groups (Fig 2). After reperfusion, control dogs exhibited little recovery of contractile function, and 4 hours after restoration of flow, the previously ischemic region was still dyskinetic, indicating severe myocardial stunning. Compared with controls, however, dogs treated with EHNA+NBMPR exhibited a significant improvement in the recovery of function, which was evident as early as 30 minutes after restoration of flow and was sustained throughout the rest of the reperfusion phase (Fig 2). Thickening fraction (expressed as percentage of baseline) was significantly greater than in control dogs at 30 minutes (−13.8±11.8% vs −50.1±7.4%), 1 hour (−12.2±12.8% vs −42.0±6.5%), 2 hours (−12.6±11.3% vs −39.6±6.0%), 3 hours (0.0±14.3% vs −36.3±5.8), and 4 hours (15.7±15.1% vs −32.8±6.8%) of reflow.
As shown in Fig 3, in control dogs wall thickening after reperfusion was directly related to collateral flow during coronary occlusion (r = 0.63). ANCOVA demonstrated that in dogs receiving EHNA+NBMPR, this relation was altered, so that for the same level of collateral flow, thickening fraction after reperfusion was greater than in controls. These results indicate that the enhanced recovery of wall thickening effected by the drugs was independent of any differences in blood flow during ischemia.

**Phase II**

Analysis of data was carried out in eight control and nine treated dogs. TTC staining confirmed the absence of irreversible damage in all animals.

The measurements of adenosine nucleotides and nucleosides are summarized in Table 4 and in Figs 4 through 6. In both the control and treated groups, myocardial ATP decreased by approximately 40% at the end of the ischemic period and remained depressed after reperfusion (Fig 4). There were no significant differences in ATP between the two groups at any time point. Similarly, no significant differences were noted with respect to ADP or AMP levels (Table 4). Major differences, however, were noted with respect to nucleoside contents. In control dogs, ischemia was associated with a marked accumulation of inosine (Fig 6), whereas the increase in adenosine was much less pronounced (Fig 5) (the inosine-to-adenosine ratio was ≈ 6:1). Reperfusion was associated with a rapid washout of both inosine and

### Table 2. Hemodynamic Variables in Phase I of the Study

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<th>Baseline</th>
<th>Preocclusion</th>
<th>Occlusion</th>
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<th>2 h</th>
<th>3 h</th>
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<tr>
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<td>165±4</td>
<td>166±4*</td>
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<td>168±5*</td>
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<td>2137±148*</td>
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<td>1843±133†</td>
<td>1789±130†</td>
<td>1722±131†</td>
<td>1752±136†</td>
<td>1635±120†</td>
</tr>
<tr>
<td><strong>dP/dt min, mm Hg/s</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EHNA+NBMPR</td>
<td>3517±137*</td>
<td>3520±148</td>
<td>2990±130**</td>
<td>3003±132**</td>
<td>3290±150*</td>
<td>3087±148**</td>
<td>3189±78*</td>
<td>2785±143†</td>
</tr>
<tr>
<td>Control</td>
<td>2802±208</td>
<td>3058±337</td>
<td>2385±169†</td>
<td>2535±161†</td>
<td>2600±172</td>
<td>2599±189</td>
<td>2504±194</td>
<td>2483±192†</td>
</tr>
</tbody>
</table>

HR indicates heart rate; bpm, beats per minute; EHNA, erythro-9-(2-hydroxy-3-nonyl)adenine; NBMPR, 6-(4-nitrobenzyl)-mercaptopurine; SAP, systolic arterial pressure; MAP, mean arterial pressure; RPP, rate-pressure product (HR×SAP/1000); LAD flow, left anterior descending coronary artery blood flow; LV dP/dt max, maximal rate of left ventricular pressure rise; and LV dP/dt min, maximal rate of left ventricular pressure fall. Baseline measurements were taken before administration of treatment drug (saline for controls); preocclusion measurements were taken 5 minutes before coronary occlusion.

Values are mean±SEM. *P<.05 vs control; †P<.05 vs baseline.

### Table 3. Occluded Bed Size and Regional Myocardial Blood Flow in Phase I of the Study

<table>
<thead>
<tr>
<th></th>
<th>Occluded Bed</th>
<th>Ischemic Zone Flow, mL·min⁻¹·g⁻¹</th>
<th>Nonischemic Zone Flow, mL·min⁻¹·g⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g</td>
<td>% LV Weight</td>
<td>Epicardial</td>
</tr>
<tr>
<td>EHNA+NBMPR</td>
<td>19.1±1.1</td>
<td>21.2±1.4</td>
<td>0.18±0.04</td>
</tr>
<tr>
<td>Control</td>
<td>17.2±1.0</td>
<td>21.6±1.1</td>
<td>0.17±0.03</td>
</tr>
</tbody>
</table>

EHNA indicates erythro-9-(2-hydroxy-3-nonyl)adenine; NBMPR, 6-(4-nitrobenzyl)-mercaptopurine; and LV, left ventricular. Data are mean±SEM. *P<.01 vs control.
adenosine, so that by 30 minutes of reperfusion, the levels of these nucleosides were not significantly different from baseline (Figs 5 and 6). Compared with control dogs, the animals treated with EHNA+NBMPR exhibited a much larger increase in adenosine (6.07±1.47 vs 1.03±0.16 nmol/mg protein, P<.05) and a much smaller increase in inosine (0.52±0.27 vs 3.04±0.54 nmol/mg protein, P<.05) during ischemia (Figs 5 and 6), indicating effective inhibition of adenosine deaminase by EHNA. The inosine-to-adenosine ratio noted in controls during ischemia (=6:1) was completely reversed in treated dogs (=1:6). Thus, the main metabolite of ATP at the end of ischemia was inosine in control dogs and adenosine in treated dogs. Furthermore, in treated dogs adenosine levels remained considerably higher than in control dogs for up to 60 minutes of reperfusion (Fig 5), indicating effective inhibition of nucleoside transport proteins by NBMPR.

Before ischemia, hypoxanthine and xanthine were low or undetectable (Table 4). A transient rise in myocardial xanthine was observed during ischemia. Neither of these bases was detectable in the myocardium during reperfusion.

Discussion

This study demonstrates that the combined administration of an adenosine deaminase inhibitor (EHNA) and a nucleoside transport blocker (NBMPR) is remarkably effective in augmenting myocardial adenosine during ischemia as well as after reperfusion in vivo. The high inosine-to-adenosine ratio present in the myocardium of control dogs at the end of ischemia (=6:1) was completely reversed by EHNA+NBMPR (=1:6), resulting in a sixfold increase in adenosine content, and the myocardial levels of adenosine continued to be much greater in treated dogs for up to 1 hour after reperfusion. The present study further demonstrates that this augmentation of adenosine results in a significant and sustained attenuation of postischemic contractile dysfunction. This beneficial effect took place soon after reperfusion (within 30 minutes) and was maintained for the subsequent 3.5 hours despite the fact that the administration of EHNA and NBMPR was discontinued 15 minutes after reflow. Importantly, the present study demonstrates that the enhanced recovery effected by EHNA+NBMPR cannot be attributed to nonspecific actions on hemodynamic variables or coro-
Fig 3. Scatterplot of the relationship between systolic thickening in the ischemic/reperfused region at 4 hours of reperfusion and transmural collateral blood flow during coronary occlusion in treated (solid circles) and control (open circles) dogs. A positive relationship is noted in control dogs (\( r = -58.2\pm206.0x, r = 63 \)). This relationship is altered by EHNA+NBMPR treatment (\( r = -28.2\pm319.7x, r = 0.56 \)) so that at any given collateral flow, treated dogs exhibit greater wall thickening than control dogs. ANCOVA demonstrated that the two regression lines were significantly different (\( P < .001 \)) and that at comparable flows, thickening fraction in treated dogs exceeded that in control dogs by an average of 45%. EHNA+NBMPR is erythro-9-(2-hydroxy-3-nonyl)adenine plus 6-(4-nitrobenzyl)-mercaptopurine ribonucleoside.

<table>
<thead>
<tr>
<th>Table 4. Myocardial Content of ADP, AMP, Hypoxanthine, Xanthine and NAD+ in Phase II of the Study</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ADP</strong></td>
</tr>
<tr>
<td>---------</td>
</tr>
<tr>
<td>EHNA+NBMPR</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td><strong>AMP</strong></td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td><strong>Hypoxanthine</strong></td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td><strong>Xanthine</strong></td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td><strong>NAD+</strong></td>
</tr>
<tr>
<td>Control</td>
</tr>
</tbody>
</table>

ADP indicates adenosine diphosphate; AMP, adenosine monophosphate; EHNA, erythro-9-(2-hydroxy-3-nonyl)adenine; NBMPR, 6-(4-nitrobenzyl)-mercaptopurine ribonucleoside; NAD+, nicotinamide adenine dinucleotide.

Values are mean±SEM and are expressed in nanomoles per milligram of protein. *P<.05 vs control; †P<.05 vs baseline.

Myocardial flow and therefore indicates a direct cardioprotective effect of these agents. Taken together, the results of this investigation indicate that augmentation of myocardial adenosine via inhibition of its degradation and washout is an effective therapeutic approach to the mitigation of postischemic dysfunction. Our findings also suggest that endogenous adenosine production during ischemia serves as an important pathophysiological mechanism that protects against stunning.

In recent years, several studies have shown that administration of adenosine produces beneficial effects in the setting of myocardial ischemia and reperfusion (reviewed in References 5 through 7). Recent observations suggest that this nucleoside enhances myocardial tolerance to ischemia via activation of adenosine A1 receptors independent of changes in ATP or coronary flow. Exogenous adenosine, however, may have limitations as a clinical therapy because this nucleoside is rapidly taken up by red blood cells and endothelial cells, so that large doses (resulting in arterial hypotension, reflex tachycardia, renal vasoconstriction, large urate burden on the kidneys, and possibly other undesirable effects) may be necessary to increase the interstitial space concentration and adequately stimulate the adenosine A1-receptors on the myocytes.5 Therefore, to avoid the undesirable side effects associated with infusion of adenosine, we sought to explore pharmacological interventions that increase endogenous adenosine levels.

To achieve this goal, we elected to administer both an adenosine deaminase inhibitor and a nucleoside transport inhibitor, notwithstanding the fact that either one used separately should have increased adenosine levels.20,23 The rationale for using this combination was that we sought to maximize the accumulation of endog-
enous adenosine by blocking its metabolism as well as its release. EHNA alone may not prevent washout of adenosine, whereas NBMPR alone may not prevent its metabolism. It should be stressed that this study was not designed to test the efficacy of one particular drug but rather to evaluate a general therapeutic approach (augmentation of adenosine). Therefore, we felt that combining two different drugs that cause augmentation of endogenous adenosine by different mechanisms would be a more conclusive means of evaluating the effect of this therapeutic approach.

Our biochemical measurements demonstrate that the doses of EHNA and NBMPR that we used were sufficient to effectively inhibit adenosine deaminase and nucleoside transport. Indeed, these doses produced a sixfold increase in myocardial adenosine content concomitant with a sixfold decrease in inosine content. The reversal of the adenosine-to-inosine ratio attests to the efficacy of EHNA in inhibiting adenosine deaminase. The persistence of elevated adenosine levels for 1 hour after reperfusion documents the efficacy of NBMPR in blocking adenosine transport. It is conceivable that there was a synergism between EHNA and NBMPR resulting in higher adenosine levels than could have been attained with either agent alone.

Our measurements of tissue nucleosides do not allow us to discern whether adenosine increased in the intracellular or interstitial compartment. The fact that EHNA+NBMPR induced vasodilation would suggest that at least some of the adenosine accumulated in the interstitial space and acted on the coronary adenosine A2-receptors. The prolonged elevation of adenosine and inosine levels after reperfusion could be compatible with either intracellular or interstitial accumulation (or both) secondary to inhibition of nucleoside transport proteins located on the membranes of myocytes and endothelial cells, respectively. Measurements of interstitial space nucleosides (eg, with microdialysis probes30) may shed light on this problem, but the question of whether intracellular nucleosides also increase will be difficult to answer. It seems unlikely that the elevation of tissue adenosine and inosine was due solely to increased concentration in the local plasma, since plasma nucleosides should be rapidly removed after reperfusion.

A major goal of this study was to separate the direct cardioprotective effects of augmenting endogenous adenosine content from the nonspecific effects of hemodynamic alterations or coronary flow increases. EHNA and nucleoside transport inhibitors are known to produce arterial hypotension and increased coronary flow,19,20,22,23 both of which could attenuate stunning independently of any direct effect on the myocytes.35 Indeed, in several previous studies19,20,22 a contribution of hypotension or hyperemia to the observed beneficial effects was not excluded. These considerations led us to
the search for a dosage of EHNA+NBMPR that would effect sufficient inhibition of adenosine deaminase and nucleoside transport mechanisms (and therefore enough accumulation of adenosine) without any contribution of arterial hypotension or coronary hyperemia to the attenuation of stunning. After evaluating seven different treatment protocols in pilot studies (see above), we adopted a protocol in which the changes in hemodynamic variables and coronary flow were such that they could not account for the enhanced functional recovery. Indeed, arterial pressure and heart rate were higher in treated than in control dogs, a difference that, if anything, should have minimized the salutary effects of EHNA+NBMPR. In our treated group, LAD flow almost tripled after reperfusion, leading to prolongation of reactive hyperemia until =15 to 30 minutes after the end of the infusion. However, coronary flow returned to baseline values by 1 hour of reperfusion, so that the attenuation of postischemic dysfunction observed between 1 and 4 hours of reperfusion cannot be explained by an increase in coronary flow. Therefore, we achieved our objective of evaluating a dose of EHNA+NBMPR that did not cause systemic hemodynamic effects or prolonged hyperemia. In addition, the control and treated groups were similar with respect to other nonspecific variables that can affect stunning such as collateral flow, ischemic zone size, body temperature, and arterial blood gases. In conclusion, our results indicate that the attenuation of stunning was due to a direct cardioprotective action of adenosine augmentation rather than to a nonspecific effect.

The beneficial effect of EHNA+NBMPR on myocardial stunning could be due to two different mechanisms: accumulation of endogenous adenosine (with resultant cardioprotection) and/or prevention of xanthine oxidase reactions (with resultant inhibition of free radical generation). With regard to the first mechanism, adenosine possesses several properties that are potentially salutary in the setting of ischemia/reperfusion9-16,39,40 (reviewed in References 5 through 7). It is a precursor of ATP and therefore may help replenish the ATP stores that were depleted by ischemia.17,18,24 However, our measurements demonstrate that in our experimental model EHNA+NBMPR had no effect on myocardial ATP levels, thereby excluding this mechanism of action. A lack of correlation between total ATP content and recovery of function in stunned myocardium has been observed previously (reviewed in Reference 37). Adenosine may increase postischemic function by increasing coronary flow (Gregg phenomenon),39 but our measurements of perfusion exclude such a mechanism of action (see above). This conclusion is corroborated by the study of Ely et al.,12 who reported beneficial effects of adenosine on ischemia/reperfusion injury in isolated hearts perfused at constant coronary flow, and by the results of Schultz et al.,41 who found that infusion of adenosine did not increase function in the stunned myocardium in open-chest pigs. Adenosine also inhibits several leukocyte functions, but there is now a consensus that leukocytes do not contribute significantly to the pathogenesis of postischemic dysfunction after a 15-minute coronary occlusion.37,42 One mechanism for protection may relate to the fact that adenosine blocks slow calcium channels,5,6 which could reduce the severity of ischemia by reducing the cytosolic accumulation of calcium. Adenosine may also decrease myocardial oxygen consumption (via reduced norepinephrine release from sympathetic nerve terminals)5,6 and at the same time may stimulate glycolysis.40 Furthermore, adenosine may decrease free oxygen radical production by decreasing lipolysis (thereby limiting generation of radicals through lipid peroxidation) and by decreasing noradrenaline release (thereby preventing catecholamine autodestruction).5,6 It is unknown which, among these actions of adenosine, is responsible for the cardioprotective effects of the nucleoside.

An alternative or additional mechanism through which EHNA+NBMPR attenuates myocardial stunning could be the prevention of xanthine oxidase–dependent generation of reactive oxygen species. There is considerable evidence that oxygen-derived free radicals play an important role in the pathogenesis of myocardial stunning.31,34,37 By preventing the deamination of adenosine to inosine and the transport of these nucleosides inside the endothelial cells, EHNA+NBMPR should decrease the substrate for the xanthine oxidase reaction and the attendant generation of ·O2- and H2O2. This (rather than adenosine accumulation) was argued by Abd-Elfattah et al17,18 to be the main mechanism of the protection conferred by EHNA+NBMPR in a canine model of global ischemia and cardiopulmonary bypass. On the basis of our present data, we cannot discern whether the protection afforded by EHNA+NBMPR in our study was due to beneficial effects of accumulated adenosine or to prevention of xanthine oxidase reactions. Further studies will be needed to identify the mechanism of action of EHNA+NBMPR. For example, since the beneficial effects of adenosine during ischemia and reperfusion appear to be receptor mediated,14,15 the combined administration of EHNA+NBMPR and of an adenosine receptor blocker could be useful in determining whether the attenuation of myocardial stunning by EHNA+NBMPR is due to adenosine-induced cardioprotection.

Previous reports have demonstrated beneficial effects of nucleoside transport and adenosine deaminase inhibitors, used singly or in combination, in various models of myocardial ischemia/reperfusion injury.19-26 The vast majority of these studies, however, were conducted in models of global rather than regional ischemia. Flameng et al19 pretreated dogs with oral moflazone (a nucleoside transport inhibitor) before 1 hour of normothermic global ischemia followed by 30 minutes of reperfusion and observed biochemical and cytotoxic evidence of protection in addition to complete functional recovery in most of the treated dogs. These results were confirmed by Wood et al who used the same results were confirmed by Wood et al who used the same experimental approach and administered moflazone intravenously. Using the more water-soluble nucleoside transport inhibitor sulfofazine, Van Belle et al20 reported increased adenosine accumulation after 20 or 32 minutes of ischemia and throughout 15 minutes of reflow, decreased nucleoside washout, and preservation of cardiac output and rate-pressure product in isolated working cat hearts. Beneficial effects of moflazone and lidoflazone were also observed by Hugtenburg et al22 in isolated working guinea pig hearts subjected to 45 minutes of ischemia followed by reperfusion.

Similar results were obtained by investigators who used adenosine deaminase inhibitors alone or in conjunction with a nucleoside transport blocker. Zhu et al23 reported that the administration of EHNA in isolated rat hearts
subjected to 40 minutes of ischemia and 50 minutes of reperfusion decreased the incidence of ventricular fibrillation and improved resting tension, contraction amplitude, and heart rate. In dogs subjected to 30 minutes of global normothermic ischemia during cardiopulmonary bypass followed by 60 minutes of reperfusion, Abd-Elfattah et al.\textsuperscript{15} reported that treatment with EHNA + NBMPR increased tissue content of adenosine, prevented washout of nucleosides, and produced complete recovery of myocardial ATP and contractility. Using the same experimental model, Abd-Elfattah et al.\textsuperscript{18} subsequently obtained similar results with 60 minutes of ischemia and 120 minutes of reperfusion.\textsuperscript{18}

These studies\textsuperscript{17-22,25} provide evidence for a salutary effect of nucleoside transport and adenosine deaminase inhibitors in myocardial ischemia, but they cannot be extrapolated to the setting of myocardial stunning for several reasons. First, the duration of ischemia was such that some degree of irreversible tissue damage (infarction) was likely to be present. In contrast, myocardial stunning is, by definition, a fully reversible abnormality.\textsuperscript{1,37} It is therefore unclear whether the protective effects of inhibitors of nucleoside transport and adenosine deaminase observed in these studies\textsuperscript{17-22,25} were due to limitation of myocardial infarction or to alleviation of myocardial stunning. To overcome this problem, we used a period of ischemia (15 minutes) that is well known not to cause irreversible tissue damage.\textsuperscript{38} Second, because of the numerous fundamental differences between the isolated buffer-perfused heart subjected to global ischemia and the in situ blood-perfused heart subjected to regional ischemia, results obtained in the former model may not necessarily apply to the latter model. Third, the beneficial effects of moflazaine and soluflazine are difficult to interpret because these substances have calcium channel blocking properties in addition to nucleoside transport inhibitory properties.\textsuperscript{29,30} Therefore, we used EHNA and NBMPR, which are specific inhibitors of adenosine deaminase and nucleoside transport, respectively. Finally, some of these studies\textsuperscript{19,20,22} did not discern whether the protective effects of augmenting endogenous adenosine were due simply to increased blood flow to the ischemic myocardium (Gregg phenomenon) or to a direct beneficial effect of adenosine on the myocytes. In the present study we were able to separate the increased recovery of contractile function from any coronary flow or systemic hemodynamic effect.

Koke et al.\textsuperscript{26} found that myocardial stunning after a 15-minute coronary occlusion was attenuated by the combined administration of EHNA and dipyridamole in open-chest dogs. These results, however, are difficult to interpret because no measurement was reported of systemic hemodynamic variables, occluded bed size, collateral flow, or coronary flow after reperfusion. A salutary influence of adenosine deaminase inhibition on the stunned myocardium was recently demonstrated by Dorheim et al.\textsuperscript{23} These authors found that the infusion of EHNA in open-chest dogs subjected to a 15-minute coronary occlusion followed by 1 hour of reperfusion significantly enhanced recovery of contractile function; this beneficial effect was associated with a 60-fold increase in interstitial space adenosine concentration at the end of ischemia compared with a fourfold increase in the untreated control animals. Our present results agree with those of Dorheim et al.\textsuperscript{23} and expand them by demonstrating that the enhanced mechanical recovery is independent of ATP repletion, is sustained over a 4-hour reperfusion period (compared with 1 hour in the Dorheim study), and is independent of any difference in collateral flow during coronary occlusion.

Finally, it should be mentioned that protective effects in various models of myocardial ischemia and reperfusion have been reported with AICA riboside\textsuperscript{39-43} (reviewed in Reference 46), which is thought to act by augmenting endogenous adenosine and reversing the adenosine-to-inosine ratio during ischemia.\textsuperscript{39,46}

Conclusions

We have demonstrated that augmentation of adenosine results in a sustained attenuation of myocardial stunning and that this effect is not due to changes in hemodynamic variables, coronary flow, or myocardial ATP levels. These findings suggest that endogenous adenosine—which is normally produced during ischemia—plays an important pathophysiological role in protecting the myocardium against stunning. Our results also indicate that augmentation of endogenous adenosine (without exogenous adenosine administration) represents an effective therapeutic approach to the prevention of postischemic contractile dysfunction and that it acts through a direct cardioprotective mechanism.

Acknowledgments

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References

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M E Zughaib, A S Abd-Elfattah, M O Jeroudi, J Z Sun, S Sekili, X L Tang and R Bolli

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