Increased Adenosine Concentration in Blood From Ischemic Myocardium by AICA Riboside

Effects on Flow, Granulocytes, and Injury*

Harry E. Gruber, Mike E. Hoffer, David R. McAllister, Paul K. Laikind, Thomas A. Lane, Geert W. Schmid-Schoenbein, and Robert L. Engler

Morbidity and mortality from acute coronary artery occlusion may be reduced if local myocardial adenosine concentration is augmented because 1) coronary collateral blood flow during ischemia increases with adenosine infusion, and 2) granulocytes that accumulate in the microcirculation during ischemia are, to a large extent, inhibited by adenosine from generating superoxide anion free radicals, from adhering to vascular endothelium, and from damaging endothelial cells in culture. Using a cultured lymphoblast model system, we found that 5-amino-4-imidazole carboxamide (AICA) riboside enhanced adenosine accumulation during ATP catabolism. Therefore, AICA riboside pretreatment was used in canine myocardium to selectively increase adenosine concentration in the ischemic area during 1 hour of ischemia. At 5 minutes of ischemia, endocardial flow to ischemic myocardium in saline-treated and AICA riboside–treated dogs was 0.06±0.03 and 0.34±0.11 ml/min/g, respectively (p<0.01); flow to nonischemic myocardium was not affected. Ventricular tachycardia and premature ventricular depolarizations were significantly attenuated in the AICA riboside–treated dogs. Blood pressure and heart rate were not affected by AICA riboside. In venous blood from ischemic tissue, adenosine increased from undetectable levels (<0.01 μM) to 0.22±0.08 μM in saline and 1.79±0.06 μM in AICA riboside–treated dogs, respectively (p<0.001). Coronary vein inosine concentrations were greater in saline than in AICA riboside–treated dogs. In separate in vitro studies, AICA riboside did not alter the removal rate of adenosine from canine blood. Indium-labeled granulocyte accumulation was significantly less in ischemic myocardium in AICA riboside–treated compared with saline-treated dogs. In addition, adenosine, but not AICA riboside, inhibited in vitro canine granulocyte superoxide production. We conclude that AICA riboside given before myocardial ischemia augments adenosine concentration, decreases arrhythmias, decreases granulocyte accumulation, and improves collateral flow to ischemic myocardium. One of the beneficial mechanisms could be an increased production of adenosine rather than inosine from ATP catabolism that causes vasodilation and inhibition of granulocytes. We propose a new hypothesis regarding regulation of the inflammatory reaction to ischemia in the microcirculation. Adenosine, in addition to its vasodilator action, is an anti-injury autacoid that links ATP catabolism to inhibition of granulocyte adherence, microvascular obstruction, and superoxide anion formation. (Circulation 1989;80:1400–1411)

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A ugmentation of collateral blood flow to the ischemic myocardium during acute coronary artery occlusion is highly desirable because the resultant infarction should be smaller and morbidity and mortality should be reduced. Adenosine is a known coronary vasodilator. During studies in cultured human lymphoblasts, we discovered that 5-amino-4-imidazole carboxamide (AICA) riboside could augment adenosine release from cells with net ATP catabolism. We found that pretreatment of cultured cells with AICA riboside followed by treatment with 2-deoxyglucose to energy starve and to cause net catabolism of ATP resulted in augmented adenosine concentration, but AICA riboside alone did not increase adenosine release. We then measured the effects of AICA riboside on blood flow to ischemic canine myocardium. Enhanced adenosine concentration in ischemic myocardium could alter the oxygen supply demand relation by several physiologic mechanisms. First, coronary vascular resistance in ischemic areas is not minimal and flow can be augmented by adenosine infusion. Second, adenosine, which inhibits activated granulocyte generation of superoxide anion free radicals, adherence of granulocytes to vascular endothelium, and destruction of cultured endothelial cells should reduce the progressive granulocyte entrapment in the microcirculation during ischemia. Granulocytes plug capillaries during ischemia and reperfusion and cause capillary no-reflow. They are responsible for the increase in coronary vascular resistance to collateral flow that occurs during the first hour of ischemia, and experimentally induced agranulocytosis results in a progressive increase in collateral flow during myocardial ischemia. Finally, direct negative inotropic or negative chronotropic effects of adenosine would decrease myocardial oxygen requirements. Because AICA riboside increases adenosine release only in energy-deprived cells, the adenosine-mediated effects should occur in ischemic but not in normally perfused myocardium. The present study shows that AICA riboside enhances collateral blood flow and decreases arrhythmias in ischemic canine myocardium and provides evidence that granulocyte inhibition could be one of the underlying mechanisms by which these effects occur.

Methods

Lymphoblast Adenosine Release Assay

We evaluated the effects of various concentrations of AICA riboside on adenosine release by inducing ATP catabolism in cultured cells. The human lymphoblast line WI-L2 was grown in RPMI 1640 with 20% fetal bovine serum and 2 mM glutamine in a 5% CO2 humidified incubator at 37°C. Cells were counted daily with a Coulter counter (Hialeah, Florida), and densities were maintained between 1×10^6 and 1×10^6 cells/ml in 75 cm² tissue culture flasks with up to 50 ml medium per flask. Mycoplasma contamination was excluded by a biochemical assay. Cells were grown in the presence of 0–500 µM AICA riboside for 48 hours before performance of the adenosine release assay.

Cells were harvested in 50-ml conical tubes by centrifugation at 750g for 5 minutes at room temperature. The cells were washed twice by gently resuspending the pellet in 5 ml RPMI 1640 containing 10% heat inactivated (56°C for 1 hour and dialyzed three times with 0.9% NaCl) fetal bovine serum and 2 mM glutamine. The washed cells were suspended at 1×10^7 cells/ml in the assay medium, which consisted of RPMI 1640 with 10% heat inactivated dialyzed fetal bovine serum, 2 mM glutamine, 1 µM 2-deoxycoformycin, and 11 mM 2-deoxyglucose. The 2-deoxyglucose was made equimolar with glucose to gradually induce ATP catabolism, and the 2-deoxycoformycin was present to inhibit adenosine deaminase conversion of adenosine to inosine. After resuspension of the cells in assay medium, 0.6 ml was placed in 17×100-mm sterile snap-top tissue culture tubes. The tubes were previously gassed with 5% CO2 in air. Two aliquots of each experimental condition were counted on a Coulter counter in triplicate, and all experimental conditions were performed in triplicate. The tubes were sealed tightly and incubated for 4 hours in a shaking 37°C water bath. In pilot experiments, adenosine release was shown to be complete at about 4 hours.

At the end of 4 hours, the tubes were placed on ice for 10 minutes. The cells and medium were transferred to a microfuge tube and microfuged at 4°C for 1 minute. Then, 0.5 ml supernatant was removed and placed in a microfuge tube containing 50 µl ice-cold 4.4N perchloric acid. The tubes were vortexed and placed on ice for 10 minutes. They were then microfuged for 1 minute, and the supernatant was transferred to 16×100-mm glass tubes containing 1.1 ml (two times the aqueous volume) of alamine/freon (2.45 g alamine brought to 12.5 ml with 1,1,2,3-trichloro-1,2,2 trifluoroethane). The mixture was vortexed vigorously for 1 minute and centrifuged at 1,500g for 2.5 minutes at room temperature. The aqueous phase was removed and frozen at −20°C until assayed for adenosine.

Adenosine was quantified in the extracted samples by high-performance liquid chromatography (HPLC). Extracted samples (100 µl) were loaded onto a C-18 micro Bondapak (3.9 mm×30 cm) reverse-phase column equilibrator (Waters Instruments, Rochester, Minnesota) with 4 mM potassium phosphate buffer, pH 3.4, 60% acetonitrile in water (95:5, vol:vol), and were eluted isocratically at a flow rate of 1 ml/min. Adenosine was eluted at 8–10 minutes, and its identity was confirmed by spiking samples with adenosine standards and reanalysis on HPLC or by reanalysis after adenosine cleavage with adenosine deaminase. The adenosine cleavage was performed by incubating 1 µl adenosine deaminase (ADA-VI, 1000 U/ml) (Sigma Chem-
ical, St. Louis, Missouri) with 110 μl extracted sample at 37°C for 15 minutes. Eluant from the column was monitored by continuous in-line light absorption at 254 nm.

**Flow Studies**

Mongrel dogs were anesthetized with phenobarbital i.v. (25 mg/kg), and the heart was exposed through a left thoracotomy. A snare was placed around the left anterior descending coronary artery. The anterior coronary vein was cannulated with a segment of polyethylene tubing of appropriate diameter and minimal length. Arterial pressure was monitored through a femoral artery catheter, and a catheter was placed in the left atrium through its appendage. Pressure and the electrocardiogram were recorded continuously on an eight-channel physiologic recorder (Gould, Cleveland, Ohio). Coronary venous blood (1.0 ml) from the vein adjacent to the occluded artery was collected into ice-cold 2N perchloric acid (0.5 ml) before drug infusion, 5 minutes before occlusion of the left anterior descending coronary artery, at various times during ischemia, and 1 minute after reperfusion. Regional myocardial blood flow was measured with 15-μm radiolabeled spheres infused into the left atrium with reference blood withdrawn from the femoral artery.14 Potassium-arrested hearts were rinsed in saline and sliced into four or five sections from apex to base perpendicular to the long axis. Myocardium was divided into endocardial and epicardial halves and normal and central ischemic areas using postmortem dye injection in the flow studies. Marginal tissue with overlap of normal and ischemic areas was excluded. Tissue was placed in tared tubes, weighed, and counted in an Autogamma counter (Packard Instruments, Downers Grove, Illinois). Overlap between channels was resolved by solution of simultaneous linear equations obtained by counting pure standards of a known number of spheres. Five saline- and six AICA riboside–treated animals survived the procedure. Animals with ventricular fibrillation were excluded. Lidocaine was not used in any animal in this series of experiments because lidocaine reduces granulocyte endothelial adherence.

**Rhythm**

Arrhythmias were counted from the real-time record or from playback of data recorded on an eight-channel FM tape.

**Purine Nucleoside Measurements of Blood**

Venous blood for purine nucleoside determinations was collected into microfuge tubes prefilled with ice-cold 2N perchloric acid, vortex mixed immediately, and placed on ice. After extraction, as described above, adenosine, inosine, and AICA riboside levels were determined by reverse-phase HPLC. For inosine and AICA riboside, loading buffer A was 10 mM KH₂PO₄ brought to pH 3.5 by addition of 1 M phosphoric acid. Eluting buffer B was 10% acetonitrile in water. After equilibrating the column with buffer A, 50 μl of a sample fourfold diluted in buffer A was injected with an IBM LC/9505 Automatic Sample Handler. The following gradient was then used. At 10 minutes, buffer B was gradually increased throughout a period of 10 minutes to 9%, at 24 minutes the percent buffer B was brought back to zero during a 1-minute period, and at 50 minutes, the program was ended. Inosine was eluted at approximately 27 minutes and AICA riboside at 15 minutes. For adenosine, buffer A was 10 mM KH₂PO₄ brought to pH 2.9 by addition of 1 M phosphoric acid. Buffer B was 10% acetonitrile in water. Fifty microliters of an undiluted sample was injected every 35 minutes. An isocratic program was used with 10% buffer B. Adenosine was eluted at approximately 18 minutes. Peaks were identified by spiking control samples with standards. For adenosine and inosine, values were corrected for background by subtracting the value obtained from reanalysis after digestion with adenosine deaminase or purine nucleoside phosphorylase (calf spleen, Sigma Chemical), respectively. Adenosine assay sensitivity is 0.1 μM with 10% variation on duplicate samples.

**Protocol**

Saline or 100 mM AICA riboside in saline was randomly selected for infusion at a rate of 1 ml/min into the femoral vein for 45 minutes before coronary artery occlusion and continued during myocardial ischemia. This dose was selected based on the results of the lymphoblast release assay, the pharmacokinetic data available, and the assumption that a concentration of 50 μM AICA riboside should result in higher in vivo phosphorylated derivatives than in rapidly dividing lymphoblasts. The left anterior descending coronary artery was occluded for 1 hour with a Schwartz clip. Radiolabeled microspheres (⁶⁵Zn, ¹¹⁵Sn, ⁹⁵Nb, or ¹⁰⁷Ru) selected randomly were injected at 5 and 55 minutes of ischemia for flow measurements. After 60 minutes of ischemia, the clip was removed and reperfusion was allowed for 5 minutes before arrest with potassium chloride. Coronary venous adenosine levels were measured 45 and 5 minutes before ischemia, at 1, 10, 20, 30, 50 minutes of ischemia, and after 1 minute of reperfusion.

**Granulocyte Accumulation**

Nine dogs were anesthetized and prepared as in the flow studies. Immediately after anesthesia, 50 ml blood was drawn into 6,000 U heparin and 40 ml hextend. Granulocytes were isolated and labeled with ¹¹¹In as previously described. Labeled cells were injected into the animals 45 minutes before ischemia. Dogs were randomized to saline or AICA riboside treatment and underwent infusion as in the flow studies. The coronary artery was occluded for 1 hour, and flow was measured at 5 minutes and 55 minutes of ischemia. After euthanasia, the heart was sectioned and counted as above for flow and ¹¹¹In content. In the granulocyte accumulation studies, myocardium was...
divided by gross visual estimation and confirmed as being ischemic by regional flow measurements less than 50% of normal myocardium remote from the ischemic region. We did not use postmortem dye injection in the granulocyte accumulation studies because of concerns that these injections would alter the granulocyte content of the normal and ischemic tissue unevenly. Blood samples for 111In and complete blood counts were drawn before cell injection, before ischemia, and at 15, 30, and 60 minutes of ischemia. Tissue granulocyte content was calculated as follows: Granulocyte content = [111In counts/g tissue] (granulocytes/μl blood) x [111In counts/μl blood]. Blood granulocyte count and 111In activity at 60 minutes of ischemia were used because the granulocyte count and blood 111In were relatively constant during the hour of ischemia.

Granulocyte Superoxide Anion Generation

The effect of adenosine and AICA riboside on the ability of dog granulocytes to generate superoxide anion was measured by the cytochrome c reduction assay as previously described. Blood from dogs was mixed in a 9:1 volume ratio with 3.4% sodium citrate. Erythrocytes were removed allowing sedimentation for 45 minutes by incubating the blood at room temperature with a 1:1 volume ratio of 6% hydroxyethyl starch in 0.85% saline (McGaw Labs, Irvine, California). The granulocytes were separated from mononuclear cells using a Ficoll-Hypaque gradient, and the remaining erythrocytes were lysed by hypotonic shock. The granulocytes were suspended in Hank’s balanced salt solution, pH 7.4, with 0.5% bovine serum albumin at a concentration of 3 x 10^6 cells/ml, with or without adenosine or AICA riboside. Assays were run in duplicate tubes that contained 1 ml granulocytes and 120 μM cytochrome c. Granulocytes were incubated with cytochalasin B, 5 μg/ml, for 5 minutes at 37°C, followed by the addition of 1 μM formyl-methionyl-leucyl-phenylalanine (FMLP) and incubation at 37°C for another 5 minutes. The tubes were placed on ice for 5 minutes, centrifuged at 450g for 10 minutes, and the absorbance of the supernatant was measured at 550 nm using a blank with bovine serum albumin. For each experimental point, additional tubes contained 30 μg/ml superoxide dismutase. Cytochrome c reduction was calculated from the difference between absorbance values of samples with and without superoxide dismutase. In a second series of studies to confirm adenosine inhibition of superoxide production using a different activator, autologous serum was activated with zymosan and used in place of the FMLP. Opsonized zymosan was prepared by incubating 50 mg zymosan A particles with fresh canine serum for 30 minutes at 37°C followed by a single wash in saline and resuspension at 10 mg/ml in saline. Polymorphonuclear leukocytes were preincubated for 5 minutes at 37°C with adenosine, followed by addition of the opsonized zymosan. Superoxide dismutase inhibitable superoxide production was read after 10 minutes at 37°C.

For each experiment, the rate of superoxide generation of unstimulated granulocytes was also ascertained as an additional control.

Adenosine Removal Assay

The rate of removal of adenosine from dog blood was measured by adding 50 μl 500 μM adenosine in saline to 5 ml dog blood previously mixed with 50 μl saline or 5 mM AICA riboside in saline. Aliquots (1 ml) were removed immediately and after 1, 5, and 10 minutes of incubation at 37°C, added to 0.5 ml ice-cold 2N perchloric acid, extracted, and analyzed for adenosine by HPLC.

Chemicals

Unless otherwise stated, all chemicals were purchased from Sigma Chemical, St. Louis, Missouri.

Statistical Analysis

Regional myocardial blood flow was tested for significant differences with repeated-measures ANOVA. Other variables were compared by Student's unpaired t test. Data are mean±SD.

Results

AICA Riboside Effect in Cultured Cells

Preincubation with increasing concentrations of AICA riboside resulted in increased concentrations of extracellular adenosine in 2-deoxyglucose–treated lymphoblasts (Figure 1). Lymphoblasts treated with AICA riboside but not 2-deoxyglucose show less than 0.1 μM adenosine concentration in the medium. Without deoxycoformycin inhibition of adenosine deaminase, no significant extracellular adenosine can be detected with or without AICA riboside, indicating that AICA riboside is probably not working through inhibition of adenosine deaminase.

Flow Studies

The whole blood concentration of AICA riboside in treated dogs immediately before coronary artery occlusion was 57.4±40.2 μM (n=6). The range was 4.4–100 μM.

Regional myocardial blood flow to the ischemic myocardium was significantly greater in AICA riboside– than in saline-treated animals (Figure 2). A similar degree of difference in flow was seen in endocardium and epicardium, and there was no significant change between 5 and 55 minutes of ischemia. AICA riboside did not alter flow to normal myocardium.

Systemic arterial pressure and heart rate at 5 and 55 minutes were not significantly different between the two groups of dogs (Table 1). Arterial blood gas content and systemic venous white blood cell counts were not significantly different between the two groups.
Electrocardiograms recorded during ischemia were analyzed for the number of premature ventricular depolarizations (PVDs) and ventricular tachycardia (VT) episodes (3 sequential PVDs with a rate >100 beats/min, Table 2). The saline-treated dogs had an average of 112.2 PVDs and 18.2 episodes of VTs during ischemia compared with 37.8 PVDs and 4.7 episodes of VTs for the AICA riboside–treated animals (p<0.01 using log transformation to correct for nonparametric distribution). One AICA riboside–treated dog (dog 3) with frequent arrhythmias had lower collateral blood flow rates and adenosine concentrations compared with the other AICA riboside–treated dogs despite an AICA riboside blood concentration of 27 μM.

**Venous Adenosine Concentrations**

The two groups of dogs showed different profiles of coronary venous adenosine concentrations over time (Figure 3). Before ischemia, none of the dogs had measurable venous adenosine (<0.01 μM) before or during AICA riboside or saline infusion. The saline-treated animals had a peak adenosine level of 0.22±0.08 μM at 10 minutes after occlusion that fell to an undetectable level by 50 minutes. In contrast, the AICA riboside–treated animals had a peak adenosine level of 1.79±0.35 μM at 1 minute of ischemia that remained elevated at 50 minutes (0.18±0.15 μM). Reperfusion resulted in no detectable venous adenosine in saline-treated animals but a significant rise in the AICA riboside–treated animals. In a separate series of dogs studied with an identical protocol, the right atrial adenosine level was undetectable at all times, but right atrial adenosine was not measured in the current experiments.

**Studies Relating to Mechanism of Action of AICA Riboside**

AICA riboside–treated dogs had a lower coronary venous inosine concentration than saline-treated dogs (Figure 4). The adenosine to inosine ratio was 69.0×10⁻³ in AICA riboside animals and 6.6×10⁻³ in controls at 10 minutes of ischemia. This implies that AICA riboside, or some metabolic product, inhibited the conversion of adenosine to inosine or of AMP to IMP.

Adenosine metabolism in canine heparinized blood was evaluated. AICA riboside was added to tubes containing canine blood and the elimination of 5 μM adenosine was compared with control tubes without AICA riboside. During a 10-minute period, elimination rates of adenosine with AICA riboside in saline or saline alone were 0.24±0.08 and 0.28±0.07 μmol/l/min, respectively (NS).

**Granulocyte Accumulation**

Granulocyte content in the ischemic endocardium was less in AICA riboside–treated dogs (1.03±0.21×10⁶ cells/g) than in saline-treated animals (1.55±0.24×10⁶ cells/g, n=9, p<0.02, Table 3). Granulocyte content in epicardium was not different in saline- and AICA riboside–treated animals. Coronary collateral blood flow during ischemia was also greater in the endocardium of AICA riboside–treated animals (0.50±0.07 ml/min/g) than in saline-treated animals (0.22±0.13 ml/min/g, p<0.001) at 5 minutes of ischemia as in the previous flow studies. However, the difference in collateral flow to epicardium was not significant (saline: 0.40±0.23; AICA riboside: 0.55±0.34, NS).

**Superoxide Generation**

Adenosine significantly decreased maximal superoxide anion generation by FMLP-stimulated canine granulocytes in a dose-dependent fashion (Figure 5). Superoxide anion generation was significantly inhibited by 1.0 μM adenosine, a concentration between the peak values measured in the saline- and AICA riboside–treated animals. AICA riboside at concentrations up to 500 μM had no effect on stimulated granulocyte superoxide anion formation and did not alter the inhibitory effect of adenosine on superoxide anion generation. The rate of superoxide anion generation in resting cells was not affected by AICA riboside. In response to zymosan-activated serum, adenosine also inhibited superoxide production (Table 4).

**Discussion**

AICA riboside administration before myocardial ischemia alters several consequences of ischemia and reperfusion. During ischemia, venous
Adenosine concentration is greater and inosine is less, collateral flow is greater, arrhythmias are fewer, and granulocyte accumulation is less. Furthermore, in vitro, maximal superoxide production by canine granulocytes is inhibited by adenosine but not by AICA riboside.

**Lymphoblast Assay**

We used the cell culture system to screen for agents that would alter the ratio of various nucleosides released during net ATP catabolism. We sought a drug that would increase the amount of adenosine for a given stimulus to ATP depletion. Performing all experimental screening assays with deoxycoformycin in control and drug tubes implies that the drug would have a site of action other than as an adenosine deaminase inhibitor.

**AICA Riboside and Adenosine Effects**

Our findings suggest that increased adenosine concentration with subsequent vasodilation and inhibition of granulocyte function is one of the beneficial mechanisms of AICA riboside present before and during ischemia. AICA riboside administration

<table>
<thead>
<tr>
<th>TABLE 1. Effects of Saline and AICA Riboside on Heart Rate and Blood Pressure</th>
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<tbody>
<tr>
<td>Saline treatment</td>
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<tr>
<td>------------------</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
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<tr>
<td>Blood pressure (mm Hg)</td>
</tr>
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</table>

All values are mean±SD. No significant differences between saline and AICA riboside were found. AICA, 5-amino-4-imidazole carboxamide.
alone did not alter blood pressure, heart rate, or resting coronary blood flow, indicating that our main findings are not due to some direct effect of AICA riboside or to changes in these determinants of myocardial oxygen demand. Furthermore, flow was not altered in the normally perfused bed, suggesting that a metabolic product produced during normal perfusion did not affect vascular resistance. We cannot exclude the existence of some other direct-acting metabolite of AICA riboside produced only during ischemia or direct inhibition of some other inflammation process (i.e., thromboxane synthesis, leukotrienes, etc.). Finally, AICA riboside alone is not a vasodilator and does not inhibit superoxide anion formation by canine granulocytes. Again, we cannot exclude some other undiscovered effect or product of AICA riboside that inhibits granulocytes, but increased adenosine alone would be sufficient to account for our observations.

During graded reductions in coronary blood flow, or during complete occlusion, coronary vascular resistance is not minimal, and flow can be augmented by adenosine infusion.\textsuperscript{3-5} One mechanism of this augmented flow during adenosine infusion is probably vasodilation because adenosine is a potent vasodilator and has been hypothesized to be a mediator of coronary autoregulation.\textsuperscript{1,18} AICA riboside did not increase flow to nonischemic areas presumably because adenosine concentration is not enhanced in nonischemic tissue.

### TABLE 2. Ventricular Arrhythmias During Coronary Artery Occlusion

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Arrhythmias (episodes/hr)</th>
<th>Ventricular depolarization</th>
<th>Ventricular tachycardia</th>
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<tbody>
<tr>
<td>Saline</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>1</td>
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<tr>
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<td>Mean</td>
<td>112.2</td>
<td>18.2</td>
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<td>AICA riboside</td>
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<tr>
<td>6</td>
<td>6</td>
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</tr>
<tr>
<td>Mean</td>
<td>37.8*</td>
<td>4.0*</td>
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AICA, 5-amino-4-imidazole carboxamide; \( p<0.01 \) from saline-treated dogs by Student’s \( t \) test on transformed data.
Increased collateral flow can also be a result of adenosine inhibition of granulocyte-mediated mechanisms of increased vascular resistance. Canine granulocytes, like human granulocytes,6,7 showed minimal inhibition of superoxide production (not significant) at the coronary venous adenosine level found in control animals but showed significant inhibition at the concentration measured in AICA riboside-treated animals. Inhibition of neutrophil superoxide production by adenosine was seen in response to FMLP, a weak but significant activator of canine neutrophils,19 and in response to zymosan-activated plasma. By inhibiting superoxide anion generation from granulocytes, adenosine should increase the local concentration of endothelium-derived relaxing factor, which is a direct vascular smooth muscle dilator.20 Leukocyte capillary plugging, which causes mechanical obstruction and capillary no-reflow, is primarily dependent on the adherence of granulocytes to vascular endothelium.21,22 Adenosine inhibits human granulocyte adherence to and injury of endothelial cells.7 In addition, vasoconstrictors produced by granulocytes10,22,23 could be suppressed by adenosine or be counteracted by adenosine-induced vasodilation. The degree of increase in collateral flow we observed may be significant in altering the ultimate extent of myocardial necrosis24 because collateral flow is an important determinant of myocardial infarct size.25

The decreased arrhythmias in AICA riboside-treated dogs could also be due to augmented adenosine. Increased collateral blood flow should reduce ischemia-mediated arrhythmias. In addition, adenosine could have a direct inhibitory effect on arrhythmias by stimulating myocyte adenosine receptors. Finally, adenosine should reduce granulocyte superoxide initiation of lipid peroxidation or possibly granulocyte release of phospholipase; both superoxide26 and lysosphospholipids27 are arrhythmogenic agents. Granulocyte depletion has been shown to reduce arrhythmias during myocardial ischemia.10,28

Granulocyte Accumulation

Direct evidence of granulocyte inhibition in vivo in this model is provided by the11 In studies that suggest that some AICA riboside effect prevented granulocyte accumulation. The decrease in granulocyte-endothelial adherence caused by aden-

**FIGURE 4.** Plot of coronary venous inosine levels (mean±SD) measured by high-performance liquid chromatography from the same blood samples that were used to measure adenosine levels in Figure 3. Saline-treated (●) and AICA riboside-treated (○) dogs.

**TABLE 3.** Granulocyte Content in Ischemic Myocardium of Dogs Treated With AICA Riboside or Saline

<table>
<thead>
<tr>
<th></th>
<th>Endocardium</th>
<th>Epicardium</th>
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</thead>
<tbody>
<tr>
<td>Ischemic area</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>1.55±0.24</td>
<td>1.69±0.39</td>
</tr>
<tr>
<td>AICA riboside</td>
<td>1.03±0.21*</td>
<td>1.07±0.16</td>
</tr>
<tr>
<td>Normal area</td>
<td></td>
<td></td>
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<tr>
<td>Saline</td>
<td>1.58±1.12</td>
<td>1.76±0.70</td>
</tr>
<tr>
<td>AICA riboside</td>
<td>1.37±0.34</td>
<td>1.34±0.37</td>
</tr>
</tbody>
</table>

All values are cells×10⁶/g wet wt (mean±SD).
AICA, 5-amino-4-imidazole carboxamide.
*p<0.02 saline- vs. AICA riboside–treated dogs.
Adenosine Concentration, uM

Figure 5. Bar graph of effect of adenosine on superoxide dismutase inhibitable cytochrome c reduction by formyl-methionyl-leucyl-phenylalanine–stimulated canine granulocytes. Results are shown as nM superoxide/2×10^6 PMN/min. R, resting, unstimulated cells; 0.0, maximal stimulation, no adenosine. Data are mean±SEM of five experiments. *p<0.05 compared with maximal stimulation, no adenosine.

osine would be predicted to inhibit granulocyte accumulation during ischemia and reperfusion because granulocyte–endothelial adherence is important to accumulation, microvascular obstruction, and microvascular damage. In addition, the greater collateral flow in AICA riboside–treated animals would be predicted to cause less granulocyte accumulation during ischemia because granulocyte content is inversely related to collateral flow in the absence of reperfusion. In ischemic myocardium in AICA riboside–treated animals in which adenosine release persisted throughout ischemia, granulocyte content tended to be less than in normal myocardium. These findings could relate to the results of Go et al., who found decreased granulocyte content after 12 minutes of ischemia and reperfusion but increased content after 40 minutes of ischemia and reperfusion in untreated dogs. This biphasic response of granulocyte content is the inverse of the pattern of adenosine release from ischemic myocardium in the absence of AICA riboside treatment and is compatible with their conjecture that adenosine release might have washed out granulocytes in reversibly injured tissue.

Possible Mechanisms of Enhanced Adenosine Concentration

At the dose we used, AICA riboside pretreatment augmented adenosine concentration only during net ATP catabolism in cell cultures and in vivo. There are several possible biochemical mechanisms for the AICA riboside–mediated enhancement of adenosine concentration in blood from ischemic myocardium. Because AICA riboside does not increase adenylate pools in canine myocardium and can inhibit AMP production from IMP, increased conversion of AICA riboside itself to AMP is unlikely to be a mechanism for increased adenosine. We have shown that AICA riboside does not significantly decrease the rate of adenosine catabolism by canine blood, suggesting that the effects are not through inhibiting adenosine degradation or uptake by blood cells. Previous studies show that the rate of depletion of ATP during ischemia is not affected by AICA riboside.

Net adenosine release into blood from ischemic myocardium might be estimated as venous flow multiplied by concentration. We cannot calculate total adenosine release into the blood in these experiments because net venous outflow from ischemic tissue could not be measured due to extensive venous collaterals in canine myocardium. Considering the greater arterial collateral inflow in the AICA riboside–treated group, the results for adenosine release could be even more significant. However, the adenosine to inosine ratio in blood should not be affected by flow, and it was not altered by AICA riboside with adenosine added to whole blood in vitro. Therefore, the increased adenosine to inosine ratio in venous blood during AICA riboside–treated ischemia indicates that the catabolic path-

Table 4. Effects of Adenosine Concentration on Cytochrome c Reduction in Response to Zymosan-Activated Serum

<table>
<thead>
<tr>
<th>Adenosine concentration (μM)</th>
<th>Control cytochrome c reduction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>0.5</td>
<td>85±16</td>
</tr>
<tr>
<td>1.0</td>
<td>80±16</td>
</tr>
<tr>
<td>2.0</td>
<td>76±18</td>
</tr>
<tr>
<td>5.0</td>
<td>74±19</td>
</tr>
<tr>
<td>10.0</td>
<td>73±19</td>
</tr>
<tr>
<td>15.0</td>
<td>66±19</td>
</tr>
<tr>
<td>20.0</td>
<td>63±20</td>
</tr>
</tbody>
</table>

Values are mean±SD; n=3 for each point.
Unstimulated PMN cytochrome c reduction=0.08±0.04 nmol/10^6 PMN/min; control stimulated PMN without adenosine=1.40±0.18 nmol/10^6 PMN/min.
PMN, polymorphonuclear leukocytes.
ways for ATP were altered and suggests several possible biochemical sites of action for AICA riboside or a metabolite. AICA riboside has been shown not to inhibit adenosine deaminase in the concentrations present in our studies.\textsuperscript{35} However, AICA riboside is readily phosphorylated by adenosine kinase to AICA riboside monophosphate, especially in cardiac muscle. AICA riboside monophosphate has been reported to inhibit AMP deaminase with a $K_i$ of about 1 mM. Other possible mechanisms for altering the adenosine to inosine ratio include inhibition of 5' nucleotidase cleavage of IMP, activation of the 5' nucleotidase for AMP, inhibition of adenosine kinase, effects on myocardial or endothelial cell membrane transport, and increase in purine flux through AMP without increasing total adenylate pool size. The exact mechanism of action for increased extracellular adenosine during ATP catabolism in the presence of AICA riboside remains unknown.

\textit{Previous Studies of AICA Riboside Effects During Ischemia}

AICA riboside is an intermediate product in purine metabolism, and as such, it was originally proposed as a way to augment ATP resynthesis after ischemia and reperfusion.\textsuperscript{36} Subsequent studies failed to confirm improved repletion of ATP when AICA riboside was given after the onset of ischemia.\textsuperscript{33,34,37} One study, using a dose of AICA riboside much larger than the dose we used, found improved mechanical function during reperfusion after total (zero flow) global ischemia without an effect on ATP repletion.\textsuperscript{33} The average ischemic endocardial and epicardial flow rates in our control group are consistent with flow rates that other investigators have seen with similar, large anterior ischemic areas produced by occlusion near the origin of the left anterior descending coronary artery. However, the significantly greater flow in AICA riboside-treated dogs that we observed appears to be at variance with the conclusions of Swain et al\textsuperscript{36} who reported a nonsignificant trend toward greater flow in AICA riboside-treated animals (endocardial ischemic flow: 0.11±0.10 control vs. 0.20±0.26 ml/g/min AICA riboside, $n=15$, NS). The critical difference is that we began infusing AICA riboside 45 minutes before occlusion, whereas they began infusion after occlusion. The significant effect on flow in our studies might have occurred because of the earlier infusion. With an occluded artery before infusion, delivery would be dependent on collateral blood flow that is less than 8% of normal in our saline controls. This low flow may not deliver sufficient drug to prevent early microvascular resistance increases or permanent damage. Metabolites of AICA riboside such as the monophosphate of AICA riboside that accumulate over time and require ATP for synthesis could be necessary to augment adenosine concentration.

\textit{A New Role of Adenosine?}

These experiments support a new hypothesis regarding regulation of the inflammatory reaction to ischemia in the microcirculation.\textsuperscript{6,38} The activation of granulocytes could be one of the mediators of...
ischemic and reperfusion injury. In addition to its vasodilator action, adenosine has been proposed as an anti-injury autacoid that links ATP catabolism to inhibition of granulocyte adherence, microvascular obstruction, and superoxide anion formation (Figure 6). According to this concept, an injured cell that is still catabolizing ATP is capable of repair or survival. Such a cell, or the process that damaged it, could activate granulocytes, but the cell can prevent the consequences of granulocyte activation as long as it excretes adenosine. When the ATP is expended, or no longer being catabolized, the cell is no longer capable of preventing full activation of granulocytes. The vasodilating effects of adenosine would also counteract any vasoconstrictive effects of the ischemia or inflammation.

In the presence of AICA riboside in the present study, beneficial effects of augmented adenosine without augmented ATP catabolism could have resulted from altered pathways of ATP catabolism or from metabolism of AICA riboside itself to adenosine. Further support for the adenosine anti-injury autacoid hypothesis is provided by two recent studies that used direct adenosine infusion during reperfusion to limit ischemic and reperfusion injury. In cat intestine subjected to constant low-flow ischemia and reperfusion, injury was reduced by adenosine; direct effects on flow were excluded by the constant flow model. In a study of permanent ischemic injury, intracoronary adenosine infused only during reperfusion reduced infarct size from 40.9% to 9.9% of the area at risk. In addition, our preliminary studies have shown that pretreatment and long-term infusion with AICA riboside reduces infarct size as measured 3 weeks after permanent coronary artery ligation in rats. Although conclusive proof that endogenous adenosine exerts a beneficial effect in vivo is lacking, the evidence provides a firm rationale for specific tests of the hypothesis.

In the therapy of acute myocardial infarction, reperfusion is clearly an important way to salvage tissue, but ischemic and reperfusion injury could limit the desired benefit. Because the toxic effects of granulocytes in addition to free radicals from other sources are important mediators of reperfusion injury, augmenting the physiologic feedback control system for granulocyte inhibition may be an important intervention in improving tissue salvage. AICA riboside appears to be a compound worthy of further study in this regard, and other mechanisms should be investigated whereby AICA riboside could alter collateral flow. The ultimate usefulness of AICA riboside for the prophylactic treatment of myocardial ischemia will depend on the demonstration of clinical benefit during angina pectoris, myocardial infarction, or myocardial infarction with reperfusion.

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