Extracellular and Intracellular Actions of Adenosine and Related Compounds in the Reperfused Rat Intestine

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By using pharmacological tools, the biological actions of adenosine (ADO) were manipulated in rat intestine that had been rendered ischemic for 5 or 15 minutes and reperfused for 1 or 24 hours. With 100 μM ADO topically administered for 30 minutes after ischemia and then washed out, intestinal arteriolar blood flow (BF) and tissue ATP were restored to preoclusion levels, and histological damage was minimal at 1 hour of reperfusion. For comparison, with vehicle treatment after ischemia, BF was reduced by 50%, tissue ATP was reduced by 50%, myeloperoxidase levels in the intestine and lung were increased at least twofold, and mucosal villi were shortened and thickened after 1 hour of reperfusion. Furthermore, with vehicle treatment, both baseline BF and reactivity to endothelium-dependent (acetylcholine) and endothelium-independent (2-chloroadenosine) vasodilators were significantly depressed after 24 hours of reperfusion. In contrast, with ADO, baseline BF remained near normal, and vascular reactivity to 2-chloroadenosine and acetylcholine was preserved after 24 hours. The salutary effect of ADO on BF was reduced by simultaneous application of the antagonist 8-phenyltheophylline or the cellular uptake inhibitor dipyridamole. The nonmetabolized agonist 2-chloroadenosine, the purine precursor aminoimidazole carboxamide riboside, or dipyridamole alone all had favorable effects relative to the vehicle, but all were less potent than ADO. The conclusions are as follows: 1) Endogenous ADO modulates the inflammatory response evoked by intestinal reperfusion because aminoimidazole carboxamide riboside or dipyridamole, which increases its availability, generally had favorable effects, whereas 8-phenyltheophylline tended to have opposite effects. 2) Exogenous ADO arrests most of the inflammatory changes associated with reperfusion by mechanisms that include both extracellular (e.g., receptor-mediated vasodilation and granulocyte inhibition) and intracellular (e.g., restoration of ATP) actions. 3) The effectiveness of ADO-related compounds even when administered after ischemia attests to the practicality of salvaging ischemic bowel, at least in some conditions. (Circulation Research 1992;71:720–731)

KEY WORDS • ATP • ADP • AMP • aminoimidazole carboxamide riboside • ischemia • theophylline • dipyridamole • microcirculation • neutrophil • acetylcholine

The posts ischemic application of exogenous adenosine (ADO) to the intestine prevents the no-reflow phenomenon and reduces granulocyte infiltration, oxy-radical damage, and functional and histological evidence of mucosal destruction for the first hour of reperfusion. With pretreatment, it reduces capillary permeability changes, granulocyte adherence, extravasation, and oxy-radical production. These basic observations suggest a potential anti-inflammatory or cytoprotective role for endogenous ADO and novel therapeutic applications for ADO-related compounds.

This study examined the following three major questions in the reperfused intestine: 1) Is the cytoprotective property of exogenous ADO dependent on an extracellular or intracellular mechanism (or combination of the two)? Does endogenous ADO have similar properties? 2) Is the protection confined to the early reperfusion phase only? 3) Do ADO and related compounds restore tissue ATP depleted by ischemia? Is the local injury propagated into other organs? Does ADO attenuate these changes?

The questions were tested with a pharmacological approach that was based on the following assumptions:

1) 2-Chloroadenosine (2CA) is a nonspecific agonist at extracellular ADO receptors, but it is a poor substrate for uptake and rephosphorylation. 2) Dipyridamole (DIPYR) inhibits the cellular transport of ADO but has no direct agonist properties. 3) Aminoimidazole carboxamide riboside (AICAR) increases endogenous ADO levels by a combination of actions, but it has no direct agonist properties. 4) 8-Phenylethoppylline (8pTHEO) is an ADO-receptor antagonist. Some drugs were applied topically, and others were infused intravenously. Multiple inflammatory changes associated with reperfusion were quantified with direct observation of the microcirculation in situ, with microscopic examination of fixed specimens in vitro, and with assays for tissue ATP levels and granulocyte infiltration.
Materials and Methods

Series 1 Experiments: Intestinal Microcirculation After 5-Minute Mesenteric Artery Occlusion and 60-Minute Reperfusion (Partially Isolated Preparation)

Male Sprague-Dawley rats (180–220 g) were anesthetized with an aqueous mixture containing 13% urethane and 1% α-chloralose (1.0 ml/100 g body wt i.p.). Supplemental doses were administered when necessary (0.1 ml/100 g body wt i.v.). The trachea, femoral artery, and femoral vein were cannulated. Respiration was spontaneous on room air supplemented with O₂. Rectal temperature was continuously monitored and maintained at 36–37°C with a heat lamp. Femoral arterial blood pressure was continuously monitored with a transducer connected to a polygraph and typically ranged between 90 and 110 mm Hg for the 2–3-hour duration of an experiment.

By using video microscopy, the arterioles in the submucosa of the jejunum were observed in situ while the mucosa and serosa were each suffused (3–7 ml/min) with bicarbonate-buffered Ringer’s solutions with separately controlled temperatures, gas tensions, and pH. The surgical preparation and suffusion systems have been previously described in detail. Briefly, observations were made through long-working-distance (4–6 mm) objectives (model L20x, 0.32 numerical aperture, or model L32x, 0.40 numerical aperture, Leitz, Rockleigh, N.J.), a long-working-distance condenser (model L11, 0.60 numerical aperture, Leitz), and an inverted microscope (Diavert, Leitz) that was equipped with a trinocular head that provided coincident images to a velocimeter in series with a color television camera (model 3230, newvicon tube, Panasonic, Secaucus, N.J.). The output voltage from the velocimeter was proportional to red blood cell velocity and was continuously monitored on the polygraph. The television signal was conveyed to a video micrometer (model IV-550, For-A, Tokyo) and a high-resolution color monitor (model CT 1930V, Panasonic). The micrometer projected two lines on the display monitor that were manually positioned on the inner walls of arterioles to measure diameter. The output voltage from the micrometer was proportional to the distance between the lines and was continuously monitored on the polygraph. This system has been previously described in detail. Within certain limits, changes in intestinal arteriolar diameter and red blood cell velocity predict changes in whole organ blood flow in general and in this preparation in particular.

Ischemia/reperfusion protocol. After a 30–40-minute postsurgery stabilization period, an arteriole (resting diameter, 50–70 μm) that originated from a small mesenteric artery was selected for study. These “first-order arterioles” were tested for spontaneous vasomotor tone with addition of ADO (100 μM, Sigma Chemical Co., St. Louis, Mo.) to the serosal suffusate. The typical response was a rapid increase in arteriolar diameter and calculated blood flow that was sustained while ADO was in the solution. On washout, diameter and blood flow returned to the pretreatment baseline within 5–10 minutes. Complete dose–response data have been published. Approximately 10% of the experiments were terminated because intestinal motility was excessive, spontaneous vasomotor tone was absent, or systemic arterial blood pressure decreased below 70 mm Hg.

Either DIPYR (10 μM, Sigma), 8pTHEO (10 μM, Sigma), or AICAR (100 μM, Genisia Pharmaceuticals, San Diego, Calif.) was topically applied to the serosa via the bicarbonate-buffered vehicle. After a 10-minute equilibration period, a rubber ligature around the small mesenteric arteries was tightened until all blood flow stopped in the microcirculation. After 5 minutes, the ligature was released. Approximately 10% of the preparations were discarded because there was no reperfusion when the ligature was released. At the time of reperfusion, ADO (100 μM) was topically applied via the vehicle, DIPYR, or 8pTHEO suffusates, or 2CA (10 μM, Sigma) was topically applied via the vehicle suffusate. The substances remained in the suffusate for the first 30 minutes of reperfusion and were then washed out. Suffusion continued for an additional 30 minutes with the vehicle only (total reperfusion time, 60 minutes).

The suffusate concentration of DIPYR that produced cellular uptake inhibition (10 μM) and that of 8pTHEO that produced receptor antagonism (10 μM) were determined in previous studies and verified in a few pilot experiments (see “Results”). If the concentrations of DIPYR or 8pTHEO exceeded 10 μM, baseline blood flow was altered, which would have confounded interpretation of the results. At a concentration of 100 μM, AICAR had no effect on baseline blood flow; that dose was based on that used in previous studies. The concentrations of 2CA (10 μM) and ADO (100 μM) that produced half-maximal vasodilations were determined in previous studies and verified in a few pilot experiments (see also Figure 5).

In all experiments, the mucosa was suffused with the vehicle alone because previous work showed that the mucosa is relatively impermeable to ADO and its analogues.

Histology. Tissue samples were harvested at the end of some of the experiments and were preserved in an aqueous solution of 1.5% glutaraldehyde and 1% paraformaldehyde for 15 minutes. The fixed tissue was stored in 10% buffered formalin for 12–24 hours and then embedded, sectioned, and stained with hematoxylin and eosin, according to standard light microscopy techniques. Villus length, villus width, crypt depth, and mucosa thickness were measured using the video microscope system.

Series 2 Experiments: Intestinal Microcirculation 24 Hours After an Intact Intestinal Loop Had Been Rendered Ischemic for 15 Minutes

Ischemic injury and reperfusion (±treatment) were done on day 1, and the awake rat was returned to the vivarium. The next day, the previously ischemic loop was isolated, and the microcirculation was observed. On day 1, fasted rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.). Respiration was spontaneous, and supplemental O₂ was delivered via nose cone. Under sterile conditions, a 2–3-cm intestinal segment with intact innervation and vascular supply was gently drawn through an abdominal incision with sutures attached to the antimesenteric border. The mesentery was divided, and a rubber ligature was passed around the arteries supplying the tissue segment. Otherwise, there was no
surgical manipulation of the tissue. The animal was positioned laterally to prevent any tension in the mesentery or the loop of the intestine and to allow unrestricted movement of the intestinal contents through the loop.

The tissue was immersed in a glass chamber containing 0.5 mM MOPS–buffered normosol containing (mM) NaCl 90.0, KCl 5.0, CaCl2 2.0, MgCl2 1.5, sodium glutonate 23.0, and sodium acetate 25. Solution pH averaged 7.38±0.01 (n=54). Tissue temperature was continuously monitored and maintained at 36–38°C by adjusting the flow rate (3–7 ml/min) of the heated bath solution.

The ligature around the small mesenteric arteries was tightened for 15 minutes and then released. For the first 30 minutes of reperfusion, the bath contained either vehicle (MOPS buffer), AICAR (100 μM) in vehicle, or ADO (100 μM) in vehicle. In two other groups, for the first 30 minutes of reperfusion, either saline or AICAR in saline was administered intravenously (10 mg/kg bolus plus 5 mg/kg per hour), but the tissue itself was exposed to the vehicle only. The last group served as a sham-injured control. At the end of treatment, the bowel loop was returned to the abdominal cavity, which was then sutured closed. The awake animal was returned to the vivarium for 24 hours, where it was allowed free access to water.

The next day, the animal was anesthetized with chloralose/urethane. The loop of bowel from the previous day was easily recognized by locating the divided mesentery, and the microcirculation was prepared for observation using the same method described in series 1. After a postsurgery stabilization period, either acetylcholine (0.1–1,000 μM, Sigma), 2CA (0.1–100 μM), or ADO (0.1–1,000 μM) were added (in random order) to the sulfosate.

**Series 3 Experiments: Biochemical Assays 60 Minutes After Intact Intestinal Loops Had Been Rendered Ischemic for 15 Minutes**

The intact intestinal loop preparation was the same as that used in series 2 (day 1). In one set, ATP metabolites were assayed. After a 15-minute occlusion, either ADO (100 μM), AICAR (100 μM), or 2CA (10 μM) was topically applied via the tissue bath for the first 30 minutes of reperfusion only. After an additional 30 minutes of suffusion with the vehicle only, intestinal tissue samples were analyzed for ATP, ADP, and AMP using high-performance liquid chromatography (HPLC). In a second set, myeloperoxidase levels in the lung and intestine were assayed as an index of granulocyte infiltration. One group received a bolus of 8pTHEO (0.5 mg/kg i.v.) before the 15 minutes of ischemia; a second group received a bolus of 8pTHEO (10 mg/kg) followed by an AICAR infusion (0.5 mg/hr i.v. for 30 minutes) during the first 30 minutes of reperfusion; a third group received intravenous AICAR alone; a fourth group received intravenous saline alone for the entire study period; and a fifth group received a sham injury and intravenous saline. After 60 minutes of reperfusion, tissue myeloperoxidase was measured in the lung and intestine using slight modifications of previously described methods.

**Details for HPLC experiments.** The intestine was rapidly flushed with −4°C vehicle, and two samples were harvested within 5–10 seconds with Wollenberger tongs cooled in liquid nitrogen. The samples were extracted with a perchloric acid method. Briefly, frozen tissue specimens were weighed, pulverized, and homogenized (Polytron, Brinkmann Instruments, Inc., Westbury, N.Y.) at −4°C in 6% perchloric acid (50 mg tissue per milliliter of solvent). The protein was precipitated for 30 minutes at −4°C. The suspension was centrifuged at 12,000 rpm for 15–20 minutes at −4°C. The supernatant was neutralized to pH 7 with a mixture of 1 M triethanolamine plus 3 M potassium hydroxide plus 3 M potassium bicarbonate, frozen in liquid nitrogen, and stored at −105°C.

**Solute Stability and Extraction Efficiency**

Samples stored in 1.8-ml sealed bio-freeze vials (Costar Corp., Cambridge, Mass.) at −105°C were reasayed 1–2 weeks after the initial extraction. Extraction efficiencies were determined by subjecting known amounts of reference solutions to the sample extraction procedure and were 96% for ATP, 97% for ADP, and 99% for AMP.

**HPLC System**

The chromatographic column was a MinoRPC (C3, C8 chains, silica, 5-μm particle size, 4.6×20 mm, reversed phase, Pharmacia Inc., Piscataway, N.J.). The injection loop volume was 100 μl with a single-sample manual injection port. Absorbance of the eluants was monitored with an ultraviolet wavelength detector (model UV-M, Pharmacia) at 254 nm. Column temperature was controlled at 30°C with a water jacket connected to a constant temperature circulator. Solvent composition was controlled by a computerized gradient programmer (model GP-250, Pharmacia).

**Buffer Preparation**

HPLC-grade monobasic sodium phosphate (0.2 M) plus tetrabutylammonium (0.02 M) was prepared using HPLC-grade water, and pH was adjusted to 6.0 with HPLC-grade 85% phosphoric acid (solvent A). Solvent B, 50% (vol/vol) methanol, was prepared by mixing HPLC-grade methanol with solvent A. All solvents were filtered and degassed using 0.20-μm filters (Millipore Corp., Bedford, Mass.).

**Column Equilibration, Calibration, and Regeneration**

Before assays, the column was flushed with 50% and 100% methanol followed by 100% solvent A. Reference solutions of ATP, ADP, and AMP were prepared by dissolving pure standards (Sigma) in HPLC-grade water. The column was equilibrated with sodium phosphate until the repeated response to the reference solutions differed by <5%. Five different concentrations of each reference (0.1–10 μM) were used to generate standard calibration curves. The coefficients of variation for peak areas from a calibration mixture injected daily onto the same column for a 1-year study period were as follows: ATP, 4.5%; ADP, 2.8%; and AMP, 8.9%. Peak identities were confirmed by retention times (ATP, 17.3 minutes; ADP, 11.1 minutes; AMP, 8.2 minutes), absorbance ratios at 280 versus 254 nm, and “spiking” with the appropriate standard.
methanol followed by HPLC-grade acetonitrile (Fischer) to expel organic contaminants.

**Step Gradient Formation**

The gradient was 9–14% solvent B for 10 minutes and was then isocratic for 15 minutes. After the appearance of ATP, the column was discharged (14–100% solvent B for 5 minutes) and reequilibrated with solvent A.

**Details From Myeloperoxidase Experiments**

Briefly, blood was removed from the animal for 3–5 minutes by exchange transfusion (final hematocrit, <10%) with heparinized 0.9% NaCl. Tissue samples were quickly harvested from the lung and intestine, deep frozen in liquid nitrogen, weighed, and homogenized in 0.005 M potassium phosphate (pH 7.4, Mallinkrodt, Paris, Ky.). A 7-ml aliquot of the homogenate was centrifuged (16,000 rpm for 20 minutes at 4°C). The supernatant was discarded, and the pellet was rehomogenized in 7 ml of 0.05 M potassium phosphate (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide (Sigma), which is a detergent that releases myeloperoxidase from the primary granules of the cell. The rehomogenized cell suspension was sonicated on ice for 60 seconds (microultrasonic cell disrupter, Kontes, Vineland, N.J.). The diluted sample (0.1 ml) was mixed with 0.05 M potassium phosphate containing 0.1 ml of 0.016 M 3,3',5,5'-tetramethylbenzidine (Sigma) and 0.03 M hydrogen peroxide (Mallinkrodt). The myeloperoxidase concentration in each sample was determined from the peroxide-dependent oxidation of tetramethylbenzidine. The change in absorbance was measured at 655 nm and 37°C (model DV40 spectrophotometer, Beckman Instruments, Norcross, Ga.). Absorbance was converted to units per gram of wet tissue from a standard curve, in which peroxidase (type VI, Sigma) was substituted for myeloperoxidase. Two dilutions were made of each tissue sample, and duplicate measurements were made at each dilution. Thus, there were three tissue samples per animal and six absorbance measurements on each tissue sample. Duplicate measurements of absorbance varied <3% (n=30).

**Statistical Analysis**

All experimental data points are expressed as mean±SEM. Between-treatment differences were tested with one-factor analysis of variance, and individual means were compared with nonparametric tests. Significance was assessed at the 95% confidence interval.

**Results**

**Series 1 Experiments**

The two objectives of this series were 1) to test whether endogenous ADO modulates intestinal blood flow during reperfusion and 2) to test whether the cytoprotective property of exogenous ADO depends on extracellular or intracellular mechanisms. To accomplish these objectives, the following substances were topically applied to partially isolated intestinal loops at the time of reperfusion: exogenous ADO and/or its nonmetabolized analogue 2CA, cellular uptake inhibitor DIPYR, receptor antagonist 8pTHEO, or a synthetic purine that putatively increases endogenous ADO levels (AICAR).

**Hypothesis 1A.** If endogenous ADO modulates blood flow during intestinal reperfusion, then DIPYR and AICAR should both cause blood flow to increase, whereas 8pTHEO should cause it to decrease, relative to vehicle.

**Hypothesis 1B.** If exogenous ADO modulates blood flow during reperfusion primarily by an extracellular mechanism, then its action should be similar to that of 2CA but reduced by 8pTHEO and not altered by DIPYR. Alternatively, if it acts primarily by an intracellular mechanism, then 2CA should be less potent than ADO, and the action of ADO should not be altered by 8pTHEO and reduced by DIPYR. If neither result is observed, then ADO must act by a combination of intracellular and extracellular mechanisms.

Figures 1, 2, and 3 show calculated arteriolar blood flow and statistical comparisons between various treatments in a partially isolated intestinal preparation subjected to 5 minutes of ischemia and 60 minutes of reperfusion.

The top panel of Figure 1 shows the time-dependent changes in intestinal arteriolar blood flow in groups treated with vehicle, AICAR, DIPYR, or 8pTHEO. The bottom panel shows similar data in groups treated with 2CA, ADO, ADO plus DIPYR, or ADO plus 8pTHEO.

In baseline conditions before occlusion, diameters of submucosal arterioles ranged from 60 to 65 µM, and calculated blood flow ranged from 30 to 50 ml/sec. Topical application of solutions containing either 10 µM DIPYR, 10 µM 8pTHEO, or 100 µM AICAR had no detectable effect on blood flow compared with vehicle alone. Suffusion with 100 µM ADO evoked an increase in calculated blood flow (211±8%, n=30) that was virtually identical to that evoked by 10 µM 2CA (212±7%, n=5). The hyperemia evoked by 100 µM ADO was increased an additional 30±5% (n=5) by 10 µM DIPYR or was decreased 28±3% (n=5) by 10 µM 8pTHEO, which verifies the efficacies of those concentrations.

A brisk, but transient, reactive hyperemia to 200–300% of baseline followed release of the 5-minute occlusion. Thereafter, blood flow stabilized at lower steady-state values for the first 30 minutes of reperfusion. On washout of the various substances, blood flow stabilized at new lower steady states by the end of the 60-minute reperfusion period.

Figure 2 compares the effect of the eight different treatments after 30 minutes on normalized arteriolar blood flow (i.e., relative to the respective preischemic steady-state baselines). At this point in the experiment, the various treatments were still in the suffusate solution. The left bars in the figure show that blood flow followed the potency order DIPYR>AICAR>vehicle=8pTHEO. These data implicate endogenous ADO in the postischemic reactive hyperemia response.

The right bars in Figure 2 show that blood flow remained higher than baseline as long as ADO, ADO plus DIPYR, or 2CA was in the suffusate and that the vasodilation caused by ADO was blocked by 8pTHEO. These data are not surprising, because ADO and 2CA are potent vasodilators. Although DIPYR potentiated the vasodilation caused by ADO before ischemia, it did
not potentiate the response evoked by ADO after ischemia.

Figure 3 compares the same eight treatments after 60 minutes of reperfusion (i.e., 30 minutes after washout), at which point blood flow had stabilized at a steady state with all treatments. In a few additional experiments (data not shown), blood flow did not change from this steady state for an additional 2–3 hours, which is the useful life of this microcirculatory preparation. Steady-state blood flow was influenced according to the potency order ADO > ADO plus DIPYR = ADO plus 8pTHEO = 2CA = DIPYR = AICAR > vehicle > 8pTHEO. Blood flow stabilized at the original baseline only in the group treated with exogenous ADO; in all other groups, the values were lower than the preclosure baseline. The effect of ADO was blocked by either DIPYR or 8pTHEO. Furthermore, the value after 2CA washout was lower than that after ADO washout, even though both substances evoked similar degrees of vasodilation during the 30-minute application period.

Figure 4 compares the histological changes in villus length, villus width, and total mucosa thickness. With vehicle alone, villus length and total mucosal thickness were reduced by half, whereas villus width was increased by 30%. These changes were attenuated, but not prevented, by ADO or DIPYR. The benefits with 2CA on villus length and total mucosal thickness were significantly lower than those with ADO. With 8pTHEO, all three measured values were significantly different from those with ADO.

Summary of series 1 experiments. Figures 1–4 show that 1) with no treatment, postischemic blood flow stabilized below the preclosure baseline (i.e., a no-reflow phenomenon), and mucosal villus damage was histologically present; 2) with the postischemic topical application of ADO, the no-reflow phenomenon was prevented, and mucosal damage was attenuated but not prevented; 3) this action of ADO on postischemic blood flow was reduced with the simultaneous application of DIPYR or 8pTHEO; 4) postischemic blood flow and mucosal damage were attenuated with 2CA or DIPYR, relative to vehicle, but both 2CA and DIPYR were significantly less protective than ADO; 5) AICAR attenuated, but did prevent, the no-reflow phenomenon; and 6) 8pTHEO alone aggravated the no-reflow phenomenon but had no major effect on the mucosal damage.
To accomplish this objective, either ADO or AICAR was topically applied to intact intestinal loops, or AICAR was delivered via intravenous infusion for the first 30 minutes of reperfusion on one day, and the next day the loop was partially isolated, and reactivity to structurally dissimilar vasodilators (ACh, 2CA, and ADO) was observed in the microcirculation.

**Hypothesis 2.** If either ADO or AICAR has a lasting benefit in the reperfused tissue, then intestinal blood flow and reactivity to vasoactive substances should be preserved even 24 hours after treatment.

Figures 5, 6, and 7 show vascular responses evoked by ascending concentrations of acetylcholine, ADO, and 2CA in a partially isolated intestinal microcirculatory preparation. The intact tissue had been rendered ischemic for 15 minutes and reperfused for 24 hours.

Figure 5 shows that dose-response curves to all three agonists were shifted downward and to the right in those animals treated with either topical or intravenous vehicle at the time of reperfusion, relative to those with the sham injury or those treated with topical ADO, topical AICAR, or intravenous AICAR for the first 30 minutes of reperfusion.

Figure 6 compares baseline arteriolar diameter and calculated blood flow after 15 minutes of occlusion and 24 hours of reperfusion. The top panel shows that arteriolar diameter was approximately 10 μm lower in both vehicle groups compared with the treated groups or the group with sham injury. The bottom panel shows that calculated arteriolar blood flow followed a similar pattern.

Figure 7 shows the shift in vascular reactivity caused by vehicle treatment and the benefits of ADO-related

**Series 2 Experiments**

The objective of this series was to determine whether the favorable action of ADO was transient or persistent.

**Figure 3.** Bar graph showing statistical comparisons in rats after 60 minutes of reperfusion for the data shown in Figure 1. ADO, adenosine; t, topical application; AICAR, aminoimidazole carboxamide riboside; DIPYR, diprydamole; 8pTHEO, 8-phenyltheophylline; 2CA, 2-chloroadenosine. At this point in the protocol, 30 minutes had elapsed since the different substances were removed from the sulfamate. Blood flow stabilized below the baseline with all treatments except ADO, and all treatments except 8pTHEO had a positive effect, relative to vehicle. In addition, DIPYR or 8pTHEO reduced the effect of ADO, and 2CA was less potent than ADO.

**Figure 4.** Bar graphs showing quantitative histological data after 60 minutes of reperfusion for selected rats from Figure 1. ADO, adenosine; PRE-OCCl, before occlusion; t, topical application; VEH, vehicle; DIPYR, diprydamole; 2CA, 2-chloroadenosine; 8pTHEO, 8-phenyltheophylline; n, number of rats. Samples were harvested from sites within the viewing field of the video microscopy system. After 5 minutes of occlusion and 60 minutes of reperfusion, the villus length was reduced, the width was increased, and total mucosa thickness was reduced. ADO attenuated, but did not prevent, these changes; DIPYR and 2CA had lesser positive effects. The changes after 8pTHEO were similar to those with VEH.
Reactivity to ACh

Reactivity to 2CA

Reactivity to ADO

FIGURE 5. Graphs showing dose-related vascular reactivity to acetylcholine (ACh), 2-chloroadenosine (2CA), or adenosine (ADO) after 15 minutes of occlusion and 24 hours of reperfusion in six different rat groups. AICAR, aminomimidazole carboxamide riboside. In those groups treated with intravenous (iv) vehicle or topical (t) vehicle for the first 30 minutes of reperfusion, the curves were shifted down and to the right, relative to the curve for sham injury (control). Those changes were prevented with tADO, tAICAR, or ivAICAR.

FIGURE 6. Bar graphs showing statistical comparisons between the baselines for the data shown in Figure 5. ADO, adenosine; t, topical application; VEH, vehicle; iv, intravenous application; AICAR, aminomimidazole carboxamide riboside. Values were determined after 24 hours of reperfusion in rats. Compared with sham-injured control values, both arteriolar diameter and blood flow were reduced with VEH treatment. Those changes were prevented with tADO, tAICAR, or ivAICAR.

reperfused intestine and 2) to determine the effect of ADO-related compounds on remote consequences of intestinal reperfusion injury.

Hypothesis 3A. If vasodilation alone is the primary mechanism for restoring tissue ATP levels in reperfused intestine, then 2CA should be as effective as ADO, and both should be more effective than AICAR. Alternatively, if cellular uptake of ADO is more important than vasodilation for restoring tissue ATP, then ADO and AICAR should be more effective than 2CA. To test this hypothesis, either ADO, AICAR, or 2CA was topically applied for the first 30 minutes of reperfusion. After 60 minutes of reperfusion (30 minutes after treatment stopped), tissue samples were harvested from the intestine and assayed for ATP metabolites.

Hypothesis 3B. If ADO modulates the systemic inflammatory response after intestinal reperfusion, then AICAR and 8pTHEO should have opposite effects on granulocyte infiltration in the lung after intestinal reperfusion, and the effects of AICAR should be blocked by 8pTHEO. To test this hypothesis, either AICAR, 8pTHEO, or AICAR plus 8pTHEO was delivered via intravenous infusion for the first 30 minutes of reperfusion. After 60 minutes of reperfusion (30 minutes after treatment stopped), tissue samples were harvested from the lung and intestine and assayed for the granulocyte-specific marker myeloperoxidase.

Figure 8 shows the measured levels of adenine nucleotides in various experimental conditions after 15 minutes of occlusion and 60 minutes of reperfusion in an
intact intestinal preparation. ATP decreased by >80% from 10.1±0.2 to 1.5±0.2 μg/g wet tissue wt after 15 minutes of ischemia and only partially recovered to 5.2±0.3 μg/g after 60 minutes of reperfusion with vehicle only. ATP levels after treatment with topical ADO, topical AICAR, or topical 2CA were significantly elevated (9.1±0.2, 8.2±0.2, 6.7±0.02 μg/g, respectively) relative to vehicle. The difference between ADO and 2CA was significant. There was no detectable treatment effect on the levels of ADP and AMP.

Figure 9 shows tissue myeloperoxidase levels in lung and intestine after 15 minutes of occlusion of the mesenteric artery and 60 minutes of reperfusion. Baseline levels between tissues varied almost threefold. With sham injury, myeloperoxidase in the lung and intestine averaged 4.0±0.2 and 11.4±0.4 units/g wet wt, respectively. After 60 minutes of reperfusion, myeloperoxidase increased approximately twofold in both tissues. With intravenous AICAR for the first 30 minutes of reperfusion, myeloperoxidase levels were restored to near baseline. Pretreatment with 8pTHEO completely blocked this effect of AICAR.

Summary of series 3 experiments. Figures 8 and 9 show that 1) tissue ATP levels were reduced after intestinal reperfusion and granulocytes had infiltrated the intestine and lung; 2) topically applied ADO or AICAR restored ATP levels to near normal; 3) ATP was also improved with 2CA, but the increment was significantly smaller; 4) within 60 minutes after intestinal reperfusion, granulocytes had infiltrated the intestine and lung; 5) intravenous AICAR reduced, whereas intravenous 8pTHEO potentiated, granulocyte infiltration at both sites; and 6) the favorable effect of AICAR on granulocyte infiltration was blocked by 8pTHEO.

Discussion

Three slightly different experimental designs were used to examine actions of ADO in the reperfused

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**Figures 7, 8, and 9**

**Figure 7.** Bar graphs showing statistical comparisons between the thresholds and the slopes of the linear portions of the dose–response curves for the data shown in Figure 5. ADO, adenosine; t, topical application; VEH, vehicle; iv, intravenous application; AICAR, aminoimidazole carboyxamide riboside; BF, blood flow. Vascular activity in rats after 24 hours of reperfusion is shown. Compared with the sham-injured control value, the thresholds for acetylcholine, 2-chloroadenosine, and ADO were elevated and the slopes were depressed with VEH treatment. Those changes were prevented with tADO, tAICAR, or ivAICAR.

**Figure 8.** Bar graphs showing tissue adenine nucleotide levels in rats after 15 minutes of ischemia and 60 minutes of reperfusion. ADO, adenosine; PREOCC, before occlusion; t, topical application; VEH, vehicle; AICAR, aminoimidazole carboyxamide riboside; 2CA, 2-chloroadenosine; n, number of rats. ATP decreased during ischemia and only partially recovered during reperfusion with VEH treatment. The levels were significantly enhanced by tADO, tAICAR, or t2CA treatments, but 2CA was significantly less effective than ADO.
The major action of 8-phenyltheophylline, which acts as a nonselective adenosine receptor agonist. The phenomenon of no-reflow, which occurs during reperfusion of ischemic tissue, was studied in this model.

The experimental design used 15 minutes of ischemia, intact intestinal loops, and assays of ischemia and intestinal tissue myeloperoxidase levels in rats after 15 minutes of intestinal ischemia and 60 minutes of reperfusion. AICAR, aminoimidazole carboxamide riboside; iv, intravenous; VEH, vehicle; 8pTHEO, 8-phenyltheophylline; n, number of rats. With VEH alone during the first 30 minutes of reperfusion, the levels were increased approximately twofold in each tissue. These changes were prevented by iv AICAR treatment. The effect of AICAR was blocked by 8pTHEO, which suggests that its action is linked to that of ADO.

intestine. The first design used direct observation of the microcirculation in a partially isolated intestinal loop, 5 minutes of mesenteric artery occlusion, and 60 minutes of reperfusion (Figures 1–4). The major findings were as follows: 1) The posts ischemic application of ADO reduced mucosal histological damage and improved blood flow for at least the first 60 minutes of reperfusion. This effect on posts ischemic blood flow was reduced by the simultaneous application of DIPYR or 8pTHEO, and a similar effect was not observed with an equipotent vaso dilator dose of 2CA. These results suggest that extracellular receptor-mediated mechanisms cannot completely explain how ADO prevents the no-reflow phenomenon. 2) DIPYR or AICAR attenuated (but did prevent) the no-reflow phenomenon, whereas the no-reflow phenomenon with 8pTHEO was worse than that with vehicle. These results are consistent with a role for endogenous ADO in the maintenance of blood flow for at least 60 minutes after ischemia.

The second experimental design used direct observation of the microcirculation 24 hours after 15 minutes of ischemic injury to intact intestinal loops (Figures 5–7). The major finding (continued from above) was as follows: 3) The posts ischemic application of either ADO or AICAR prevented vasoconstriction and restored vascular reactivity to endothelium-dependent and endothelium-independent vasodilators. These results suggest that the protection afforded by ADO is not transient, that this property can be exploited with AICAR, and that the site of action includes both the endothelium and the vascular smooth muscle.

The third experimental design used 15 minutes of ischemia, intact intestinal loops, and assays of ischemia and intestinal tissue after 60 minutes of reperfusion (Figures 8 and 9). The major findings (also continued from above) were as follows: 4) ADO or AICAR prevented local ATP decreases, whereas 2CA had a significantly smaller effect. These results provide further evidence that ADO has more than an extracellular action, because 2CA causes vasodilatation by a receptor-mediated extracellular mechanism, and AICAR does not. 5) AICAR prevented a twofold increment in myeloperoxidase levels in the lung and intestine, and this effect was antagonized by 8pTHEO. Assuming that myeloperoxidase reflects granulocyte infiltration and that AICAR acts by increasing endogenous ADO levels, these results show that both local and systemic inflammatory changes can be attenuated by the post ischemic application of ADO-related compounds.

**Critique**

One limitation of this study is that two different occlusion periods were used, and it was assumed that pathogenesis of the resultant reperfusion injuries was similar. In those tissues prepared for observation of the microcirculation, a 5-minute occlusion of the mesenteric artery was selected because longer periods of occlusion caused irreversible tissue damage that was refractory to most treatments. It is not surprising that long periods of ischemia will push any tissue beyond the point of salvage. On the other hand, the unusual sensitivity to only 5 minutes of occlusion reflects the experimental conditions. Consider that the tissue was subjected to the surgical trauma of dividing the microvascular network along the antimesenteric border, pulling it into a flattened sheet on the microscope stage, securing it to a foreign surface, and suffusing the mucosal and serosal surfaces with artificial solutions. Indeed, 5 minutes of vascular occlusion in the intact intestinal loops used in the series 2 and 3 experiments produced almost no detectable injury in pilot experiments. Therefore, in series 2 and 3, the vascular supply was occluded for 15 minutes. If the degree of ischemic damage produced by 5 minutes of occlusion of the partially isolated preparation was different from that produced by 15 minutes of occlusion of the intact preparation, then there could have been differences in the pathogenesis of the resultant reperfusion injuries. In that case, it may be difficult to directly compare the results from Figures 1–4 (5-minute occlusion) with those from Figures 5–9 (15-minute occlusion).

A second limitation is that a pharmacological approach was used to characterize actions of ADO. The meaning of these observations depends on assumptions regarding the actions of 2CA, DIPYR, AICAR, and 8pTHEO. Furthermore, the efficacies of these substances were judged on the basis of several arbitrary criteria, such as the dosage, the time of application, the route of delivery, and the time after treatment at which the data were collected. Since each of these criteria influences the interpretation and conclusions, each will be separately considered.

First, the concentrations of DIPYR, AICAR, or 8pTHEO applied topically or those of AICAR or...
8pTHEO applied intravenously were below the threshold for altering baseline blood flow. Higher concentrations were avoided because it is unlikely that ADO has a role in the regulation of resting intestinal blood flow.\textsuperscript{4,5} In contrast, the concentrations of 2CA and ADO were titrated to produce half-maximal vasodilation. Higher concentrations of ADO than 2CA were required to evoke an equivalent vasodilation because the parent compound is avidly taken up and rephosphorylated or deaminated, whereas the synthetic analogue is poor substrate for cellular uptake and is resistant to adenosine deaminase. Most of the ADO-related compounds had less of a cytoprotective property than ADO itself, but the efficacy of higher concentrations is unknown.

Second, pretreatment or continuous application with AICAR or DIPYR will maximize their effectiveness. This condition is important because it is impossible to interpret negative results if the effectiveness is not maximized. In the series 1 experiments, the tissue was pretreated with AICAR or DIPYR. On the other hand, the usefulness of a drug is limited if it is effective only with pretreatment. Therefore, in the series 2 and 3 experiments, the various substances were applied only after the ischemic insult. It was arbitrarily decided to limit the treatment to the first 30 minutes of reperfusion, because a large fraction of granulocyte-mediated and oxidative injury probably occurs within the early phase of reperfusion.\textsuperscript{6} Thus, maximal effectiveness of the various compounds was probably not achieved with this schedule of treatment, but the favorable results attest to the practicality of tissue salvage even when ADO-related compounds are applied after ischemia.

Third, a topical or intra-arterial route of application is ideal in many experimental situations because high local concentrations are achieved at the local site of action without confounding systemic side effects. On the other hand, it may be difficult or impossible to deliver drugs via the preferred routes in real-life situations, or it may be impossible to produce a local therapeutic concentration without undesired systemic effects. For this reason, both topical and intravenous routes were compared in this study, even though the drug may have been less effective when delivered via the intravenous route.

Fourth, the intestinal mucosa is capable of rapid healing, and there is no clear distinction between the end of injury and the beginning of repair. Indeed, the mucosa can completely regenerate within 24 hours even after massive destruction.\textsuperscript{17} Thus, it is logical to anticipate that repair would begin soon after injury. If the intestine would repair itself anyway, then a short-lasting benefit of ADO treatment might have no functional significance. In this present study, the intestine was examined for the first 60 minutes of reperfusion and after 24 hours of reperfusion. Depending on the reader's point of view, a 60-minute study period could be long enough or too short. In pilot experiments, we observed that blood flow after 120–180 minutes of reperfusion was not markedly different from those values measured after 60 minutes. Since the useful life of a microcirculatory preparation is limited even in the best cases, and especially after it has been rendered ischemic, a 60-minute study period seemed a reasonable compromise. But again, if the intestine repairs itself anyway, there is no compelling need for ADO treatment. A separate series showed residual pathology (i.e., vasoconstriction and diminished vascular reactivity) after 24 hours of reperfusion that was prevented by ADO or AICAR treatment. However, at this point, the distinction between persistent or even secondary injury versus repair is blurred. It is impossible to determine whether ADO attenuated the injury or accelerated the healing.

Thus, the limitations imposed by a pharmacological approach may restrict comparisons to the work of other investigators. However, the inherent weaknesses in the experimental design should be balanced by the strength of measuring several different variables (e.g., baseline blood flow, vascular reactivity to structurally dissimilar compounds, ATP levels, myeloperoxidase levels, and mucosal architecture) and by manipulating those variables with different combinations of structurally dissimilar, but related, compounds (i.e., ADO, AICAR, DIPYR, 2CA, and 8pTHEO).

**Interpretation**

It has been proposed that during myocardial reperfusion injury, ADO is an anti-inflammatory autacoid that links ATP catabolism to inhibition of granulocyte function, microvascular obstruction, and superoxide formation.\textsuperscript{9} These present results suggest that ADO has similar actions in the reperfused intestine. Receptor-mediated vasodilation is an important property of ADO that probably contributes to its favorable effects on the no-reflow phenomenon (Figures 1–3), mucosal destruction (Figure 4), vascular reactivity (Figures 5–7), cellular energy levels (Figure 8), and granulocyte infiltration in remote organs (Figure 9). We have shown that at doses that produce vasodilation equal to or greater than that caused by ADO, acetylcholine or prostacyclin significantly improve posts ischemic blood flow and reduce histological damage in the reperfused intestine, but the magnitude of that protection is small compared with that of ADO.\textsuperscript{1} This present study shows that, at an equipotential vasodilator dose, 2CA is less protective than ADO, and the magnitude of the initial reactive hyperemia in the various treatment groups did not correlate with the eventual steady-state value. Thus, vasodilation alone is beneficial but cannot fully explain why ADO attenuates the no-reflow phenomenon.

Infiltrating granulocytes and oxy-radicals have a major role in intestinal reperfusion injury,\textsuperscript{3,16} and ADO inhibits granulocyte-mediated oxy-radical generation by a receptor-mediated mechanism in vitro.\textsuperscript{18} We reported that these mechanisms operate at the microvascular level in vivo, because granulocyte-mediated changes in microvascular permeability in the cheek pouch\textsuperscript{19} and oxidant production in reperfused intestine\textsuperscript{2} are both potentiated by the ADO receptor antagonist 8pTHEO and because ADO reduced granulocyte infiltration in the reperfused intestine.\textsuperscript{1} This present study showed that the action of ADO on blood flow during reperfusion was attenuated by 8pTHEO (Figures 1–3) and that the action of AICAR on myeloperoxidase levels in the lung and intestine was attenuated by 8pTHEO (Figure 9). Overall, these observations suggest that altered granulocyte function is another receptor-mediated mechanism by which ADO improves posts ischemic blood flow.
Nevertheless, if either vasodilation or inhibition of granulocyte function, or a combination of the two, had been the primary explanation for the effect of ADO in the reperfused intestine, then DIPYR should have potentiated, rather than attenuated, the action of ADO, and 2CA should have been as effective as, rather than less effective than, ADO. Another component, such as cellular uptake and rephosphorylation of ATP precursors, must contribute to the protective mechanism. Indeed, this study showed that ATP levels were higher with AICAR than with 2CA (Figure 8), even though 2CA causes vasodilation and AICAR does not.

Whether derived from exogenous or endogenous sources, extracellular ADO is avidly transported into hypoxic cells; therefore, it could partially replenish cellular ATP levels after ischemia,20 which could reduce susceptibility to reperfusion damage in the heart21,22 as well as in other tissues. On the one hand, it is possible that the functional ability of the intestine to maintain ion gradients is vulnerable to ATP depletion, because we have shown that mucosal pH decreases during reperfusion are prevented by ADO.2 On the other hand, higher adenine nucleotide levels per se cannot distinguish cause or effect with regard to a mechanism for ADO in any tissue for two reasons: 1) ATP levels do not necessarily correlate with function or cytoprotection,23–25 2) A simple improvement in oxygen supply/demand could have similar effects on cellular ATP. This present study showed higher ATP levels after 2CA treatment, relative to vehicle (Figure 8). Rephosphorylation of precursors cannot explain this result because 2CA is a poor substrate for cellular uptake at the concentrations used in these experiments; the higher ATP after 2CA must reflect improved oxygen supply/demand.

It should be emphasized that there is no unequivocal index of reperfusion injury or definition of treatment benefits. Our past and present work has demonstrated that ADO is associated with improvements in blood flow, granulocyte infiltration, oxidant injury, mucosal pH changes, and cellular energy depletion. However, reperfusion injury in any tissue is mediated by a host of granulocyte-mediated and non–granulocyte-mediated as well as oxidant-related and non–oxidant-related mechanisms. There is no reason to expect that ADO or any other substance would attenuate every change associated with reperfusion injury.

This present study is among the first to show a beneficial effect of AICAR on reperfusion injury with preischemic or postischemic application. AICAR is relatively ineffective without pretreatment in most models of myocardial reperfusion injury.23–25

The exact mechanism of action of AICAR in the intact reperfused intestine has never been studied, and it is impossible to draw any meaningful conclusion from the controversial in vitro data. For example, in one cell line, the production of adenine nucleotides from AICAR is similar to that from hypoxanthine with a half-maximal effect at 30–100 μM.27 In another, AICAR depressed purine biosynthesis, purine salvage, and uptake of extracellular ADO but increased ATP degradation.28 In a third, AICAR had a biphasic effect on growth, intracellular purine nucleotides, and DNA concentration.29 The in vivo data from heart and brain suggest rather convincingly that AICAR has low potency at ADO receptors and cellular uptake sites30 and that most of its biological actions can be attributed to the release of endogenous ADO,9 but AICAR may have antioxidant properties8,21 that would tend to reduce granulocyte accumulation and microvascular injury. We observed that 8pTHEO antagonized the action of AICAR (Figure 9), which supports the idea that ADO is at least partly involved.

These present results with AICAR have interesting implications. Ischemia/reperfusion injury to various tissues can occur after profound hemorrhagic shock/resuscitation even with adequate restoration of systemic hemodynamics. The accumulated burden of these injuries, particularly to the splanchic organs, can contribute to multiple-system organ failure.31,32 Many different pharmacological agents have been added to resuscitative fluids to correct the energy imbalance caused by ischemia or ameliorate the injury that accompanies reperfusion. In most cases, the drugs have nonspecific or systemic effects (e.g., ATP-MgCl₂) or have marked beneficial effects only with pretreatment (e.g., superoxide dismutase). It is attractive to speculate that these disadvantages could be avoided if resuscitative fluids were supplemented with AICAR. However, our initial attempts have been disappointing: no obvious hemodynamic benefits were detected for at least 2 hours when AICAR was added to the hypertonic colloid or isotonic crystalloid solutions used in the initial resuscitation of hemorrhagic shock.33 Further work is needed to determine whether AICAR and related compounds have salutary effects after hemorrhagic shock.

In summary, the concluding statement in a recent state-of-the-art review34 was as follows: “Endogenous adenosine plays a multifaceted role in the protection of the ischemic myocardium. The pharmacological use of adenosine, its analogues, or its transport and metabolic inhibitors may extend its clinical applications beyond its approved use as an agent for the termination of supraventricular tachyarrhythmias.” The results from this present study suggest that ADO may have similar multifaceted protective properties in the ischemic intestine.

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