Influence of Ribose, Adenosine, and "AICAR" on the Rate of Myocardial Adenosine Triphosphate Synthesis during Reperfusion after Coronary Artery Occlusion in the Dog

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SUMMARY. Recovery of adenosine triphosphate after myocardial ischemia is limited by the slow adenine nucleotide de novo synthesis and the availability of precursors of the nucleotide salvage pathways. We determined the adenine nucleotide de novo synthesis in the dog by infusion of [14C]glycine and the acceleration of adenine nucleotide built up by intracoronary infusion of ribose together with [14C]glycine or radiolabeled 5-amino-4-imidazolcarboxamide riboside or adenosine in the same animal model and with the same dosage of substrates (9 mmol) in postischemic and nonischemic myocardial tissue. After 45 minutes of occlusion of a side branch of the left coronary artery, the ischemic area was reperfused for 3 hours, and needle biopsies were taken for biochemical analysis. Adenine nucleotide de novo synthesis was found to be very slow (1.5 nmol/g wet weight per hour). The rate was doubled after ischemia. Adenine nucleotide synthesis was accelerated 5-fold by ribose, the basic substrate of the adenine nucleotide de novo synthesis, 9-fold by 5-amino-4-imidazolcarboxamide riboside, an intermediate of the adenine nucleotide de novo synthesis and 90-fold by adenosine, a substrate of the nucleotide salvage pathway. Therefore, only adenosine infusion resulted in a measurable increase of adenosine triphosphate levels after 3 hours of reperfusion, but over a longer time period, ribose or 5-amino-4-imidazolcarboxamide riboside also can be expected to replenish reduced myocardial adenosine triphosphate faster than adenine nucleotide de novo synthesis. Studies with radiolabeled 5-amino-4-imidazolcarboxamide riboside showed significant incorporation of radioactivity into 5-amino-4-imidazolcarboxamide ribose triphosphate which had also risen measurably during 5-amino-4-imidazolcarboxamide ribose infusion, and which is not normally found in heart muscle. (Circ Res 56: 220-230, 1985)

It is a well-established fact that ischemia leads to a depletion of adenosine triphosphate (ATP) (Chang, 1938; Burdette, 1956; Berne, 1963; Gerlach et al., 1963) which is not readily reversible upon reoxygenation of tissue, especially when the coronary occlusion (or other forms of oxygen deprivation) lasted longer than 10 minutes (Danforth et al., 1960; Kammermeier et al., 1963, 1964; Isselhard et al., 1964, 1970a; Schaper et al., 1979). The slow rate of ATP synthesis after ischemia (Zimmer et al., 1973) is mainly the result of two factors. (1) The conversion of glucose-6-phosphate to ribose-5-phosphate proceeds extremely slowly in mammalian heart muscle (Zimmer and Gerlach, 1978; Ibel et al., 1982). (2) Substrates for the salvage pathways, notably adenosine, but possibly also inosine and hypoxanthine, are rapidly washed out of the tissue upon reflow and are hence lost for salvage (Katori and Berne, 1966; Fox et al., 1979).

Clinical efforts to reestablish blood flow in acute myocardial infarction by fibrinolysis (Schmutzler et al., 1966; Rentrop et al., 1981) were reported to be successful, but mechanical function of the reperfused segment often returns only after a long delay. This effect is known from animal experiments (Kloner et al., 1981). Other probable metabolic effects of ischemia and reperfusion that cannot be measured in human patients may also be dependent on ATP. A fast restitution of ATP levels during reflow after ischemia appears highly desirable. We report here in a comparative study the three most promising substances, i.e., ribose, 5-amino-4-imidazolcarboxamide riboside (AICAR), and adenosine, for replenition of purine nucleotides.

Methods
Experimental Design
Thirty-eight dogs of either sex weighing approximately 28 kg were anesthetized with subcutaneous piritramide (5 kg) and intravenous sodium pentobarbital (15 mg/kg). They were artificially respired with a Bird respirator system using a gas mixture containing 30% nitrous oxide, 70% oxygen. The oxygen concentration was adjusted to
give an arterial \( P_{O_2} \) between 100 and 120 mm Hg. The \( P_{CO_2} \) varied between 38 and 45 mm Hg. A Millar catheter tip manometer was advanced into the left ventricle via the left carotid artery, aortie blood pressure was monitored with a catheter (via the right femoral artery) that was connected to a Statham P23 pressure transducer. A venous line was established via the left femoral vein. Under fluoroscopic control, a Flexo pulmocath catheter (F4) was advanced into the descending branch of the left coronary artery (LAD) via a transfemoral guiding catheter for intra-coronary infusion. The dogs were anticoagulated at the beginning with 5000 IU heparin, intravenously, followed by the same dose after 2 hours. Left thoracotomy and exposure of the heart was performed via the 5th intercostal space and a side branch of the LAD was prepared at its origin. A snare was placed around the artery for occlusion and reperfusion. The whole preparation was allowed to stabilize for 30 minutes. After 45 minutes of occlusion of the LAD sidebranch, the ischemic area was reperfused for 3 hours. Reperfusion was documented by fluoroscopic control. Nine millimoles (1 ml/min of a 50 mM solution) of ribose (seven dogs), AICAR (seven dogs), or adenosine (eight dogs) were infused via the intracoronary catheter into the LAD proximal to the previously occluded vessel. One group with saline infusion (seven dogs) served as a control.

Radiolabeled nucleosides (5-amino-2-\( ^3H \) diazole-4-carboxamidriboside, \([^3H]AICAR\), or 2-\((^3H)\)adenosine) were added to "cold" nucleosides to obtain a specific activity of 4.5 mCi in 9 mmol total amount infused (\( n = 3 \) dogs each nucleoside).

Ribose incorporation was measured by infusion of 9 mmol ribose for 3 hours, together with 1-\( ^{14}C \)glycine (specific activity 55.4 mCi/mmol) with an infusion rate of 0.8 mCi/hr (\( n = 3 \)).

Purine de novo synthesis was measured by intracoronary infusion of 1-\( ^{14}C \)glycine (specific activity 55.4 mCi/mmol) for 3 hours with an infusion rate of 0.8 mCi/hr (\( n = 3 \)).

Needle biopsies (Tru-Cut) for biochemical analysis of nucleotides, nucleosides, and creatine phosphate were taken before any intervention, at the end of 45 minutes of regional ischemia, and after 3 hours of reperfusion. In dogs that received ribose, AICAR, or adenosine infusion, biopsies after 3 hours of reperfusion were obtained from the previous ischemic area, from nonischemic areas distal to the intracoronary substrate infusion, and from nonischemic areas that received the substrate via recirculation (posterior of the heart). The experiment was terminated after the last biopsy with 10 ml of concentrated potassium chloride, the heart was removed, sliced in bread-loaf fashion, and myocardial rings were incubated in p-nitro-blue tetrazolium chloride to exclude hearts with marked subendocardial infarctions. Since we occluded only a sidebranch of the LAD, no infarction occurred within 45 minutes of ischemia which would have occurred if the whole LAD had been occluded for the same time period.

ECC, left ventricular pressure, LV dp/dt, and aortic pressure were recorded on a direct-writing inkjet recorder. Instantaneous myocardial oxygen consumption was calculated on-line according to Bretschneider's equation (Bretschnieder et al., 1970) by a digital computer, and kept in a range between 6 and 7 ml O\(_2\)/100 g per min. In dogs receiving AICAR, blood samples from the left atrium and the inferior vena cava were obtained before ischemia and in short intervals during AICAR infusion.

### Tissue Sampling and Biochemical Analysis

#### Extraction Procedures

Biopsies (10–25 mg) were frozen immediately (2–3 seconds) in liquid nitrogen and weighed on a Kettler balance in precooled vials. The frozen tissue was homogenized in ice cold 0.6 M perchloric acid (400 \( \mu l \)) with an ultrasound homogenizer (Sonicator Cell Disruptor W-225 R) for 10 seconds. The homogenate was centrifuged at 12,000 rpm for 3 minutes, and 350 \( \mu l \) of the supernatant were neutralized with the required volume of 6 N KOH, resulting in a total volume of about 420 \( \mu l \). The supernatant was decanted and stored in liquid nitrogen until it was analyzed, within the next 3 days. Blood samples were immediately centrifuged at 3000 rpm for 5 minutes, and 200 \( \mu l \) of serum were added to 500 \( \mu l \) of 0.6 M perchloric acid. The supernatant extract was neutralized with 6 N KOH and stored at −36°C.

#### Nucleotide and Nucleoside Analyses

ATP and creatine phosphate values were determined using bioluminescence, as described by Ellis and Gardner (1980). Normal ATP values in the dog are reported to be 4 \( \mu mol/g \) wet weight with this method. ADP, AMP, IMP, NAD, and AICAR-monophosphate were separated and quantified by high performance liquid chromatography (HPLC), using a Varian 5000 gradient controller combined with a DuPont spectrophotometer and a DuPont integrator. A 50- \( \mu l \) sample of the undiluted supernatant extract was injected onto a Zorbax-NH\(_2\) column (DuPont) and developed isocratically with 5 \( \mu l\) NH\(_4\)H\(_2\)PO\(_4\) (pH 2.8) for 15 minutes, followed by a linear gradient of 5 \( \mu l\) to 750 \( \mu l\) NH\(_4\)H\(_2\)PO\(_4\) (pH 4.5) at a flow rate of 2 ml/min.

Nucleosides and AICAR were separated on a DuPont ODS column using a linear gradient of 0 to 30% methanol–water (vol/vol) within 25 minutes. Uric acid and AICAR in plasma were separated on a DuPont ODS column using a linear gradient of 30 \( \mu l\) KH\(_2\)PO\(_4\) (pH 3.5) to 30% methanol diluted in 30 \( \mu l\) KH\(_2\)PO\(_4\) (pH 3.5) within 25 minutes.

The peaks found at 254 nm were identified by comparison of retention times with external standards. Peak areas were quantified by using peaks of known concentration and expressed in nmol/g or \( \mu mol/g \) wet weight. Since Reimer (Reimer et al., 1981) showed that tissue water after regional ischemia changes in the first hours of reperfusion by only 3%, no dry weight determinations were made.

For separation of amino acids in myocardial tissue, 20 \( \mu l \) of a solution containing 800 mg o-phthalaldehyde and 200 \( \mu l \) mercaptoethanol in 10 ml methanol were added to 150 \( \mu l \) of the sample for pre-column derivatization. A linear gradient of 30 minutes was established between solution A (tetrahydroduron, methanol, 0.05 M sodium acetate, pH 5.9, 0.8/19.2/80, vol/vol/vol) and solution B (methanol, 0.05 M sodium acetate, pH 5.9, 80/20, vol/vol) on a Waters Radial Compression System using a C-18 column. Amino acids were detected with a fluorescence spectrophotometer (Kratos) at an emission wavelength of 228 nm and a 470 nm cut-off filter.

#### Analysis of \([^3H]\)AICAR, \([^3H]\)Adenosine, and \([^{14}C]\)Glycine Incorporation

Four biopsies were extracted together in one vial, as described above, to obtain larger amounts of nucleotides. Fifty microliters of the supernatant extract were injected...
onto a DuPont ODS column and developed by means of a paired ion gradient system. A linear gradient was established, using 5 mM tetrabutylammonium phosphate with 30 mM KH₂PO₄ (pH 5.5) and 2% to 40% acetonitrile within 40 minutes. This resulted in a good separation of guanosine triphosphate (GTP), AICAR-triphosphate, and ATP. There was no coelution of ADP and adenylylsuccinate which occurs with the ion-exchange method used for quantification of the nucleotides. AICAR triphosphate was identified as described by Sabina et al. (1982). We sampled AMP, ADP, IMP, ATP, and, if present, AICAR triphosphate by collecting the outflow of the HPLC column for the entire peak into scintillation vials containing 10 ml xylene scintillation fluid (Packard Instruments). Radioactivity was counted on a Packard Tri-Carb liquid scintillation counter and expressed in counts/min per 100 mg wet weight. To verify that radioactivity is not found in any other compound of the chromatogram, the column outflow of one biopsy of each experiment was sampled continuously in 30s fractions. No loss or interchange of the tritium atoms from [3H]AICAR or [3H]adenosine was detected, since the radioactivity baseline was always low and stable, and radioactivity was found only in anabolic or catabolic products of AICAR or adenosine.

For quantification of [3H]AICAR and [3H]adenosine incorporation into the nucleotides, the specific activity of the substrates in the infusate was determined by quantification of the substrate concentration by HPLC. The column outflow during the substrate peak was sampled, and radioactivity was counted in a liquid scintillation counter, as described above. Since there is no measurable AICAR content and only a minimal adenosine content in reperfused myocardial tissue, the specific activity of the infused substrates equals that of the tissue, and radioactivity incorporated into nucleotides can directly be converted into concentration.

In the experiments with [14C]glycine as a marker of the nucleotide synthesis, specific activity of myocardial tissue glycine was measured directly, by the HPLC separation method, as described above. A total of 43.3 μmol of glycine was infused intracoronary within the 3-hour period. This increases plasma glycine levels by only 2.5%, assuming a 50 ml/min flow in the LAD. Because of the dilution of the intracoronary infusate in the whole body fluid, the recirculating radioactive glycine can be neglected, as compared to the activity in the intracoronary blood, i.e., the specific activity of glycine in the peripheral venous plasma was under 3%, compared to the calculated specific activity in the intracoronary plasma. Furthermore, the specific activity of myocardial tissue glycine in myocardial areas that received [14C]glycine only via recirculation was only 7% compared to areas of intracoronary [14C]glycine infusion. Therefore, because of the rapid exchange of glycine between blood and cells (Elwyn et al., 1972), a constant tissue-specific activity can be expected within minutes of the 3-hour infusion period and can be maintained over the infusion period.

**Chemicals**

AICAR, adenosine, ribose, NH₄H₂PO₄, KH₂PO₄, tetrabutylammonium phosphate, and all nucleotides and nucleosides for HPLC-standards were obtained from Sigma Chemicals. [3H]Adenosine and [14C]glycine were made by Amersham Inc. [3H]AICAR was made to our specification by Amersham, Inc. Firefly luciferase was obtained by Lumac and creatine kinase by Boehringer Mannheim.

**Statistics**

Statistics were done by paired or unpaired t-test. All values are expressed as means ± se, unless otherwise mentioned.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Hemodynamics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline</td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>110.5 ± 8.0</td>
</tr>
<tr>
<td>Ischemia (45 min)</td>
<td>94.3 ± 5.7</td>
</tr>
<tr>
<td>Reflow (180 min)</td>
<td>102.0 ± 6.2</td>
</tr>
<tr>
<td>LVPsys (mm/Hg)</td>
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<tr>
<td>Control</td>
<td>129.0 ± 5.2</td>
</tr>
<tr>
<td>Ischemia (45 min)</td>
<td>120.0 ± 6.0</td>
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<tr>
<td>Reflow (180 min)</td>
<td>117.0 ± 5.1</td>
</tr>
<tr>
<td>MVO₂ (ml/100 g per min)</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>8.74 ± 0.41</td>
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<tr>
<td>Ischemia (45 min)</td>
<td>7.29 ± 0.43</td>
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<tr>
<td>Reflow (180 min)</td>
<td>6.98 ± 0.31</td>
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*Results (in all tables) are expressed as means ± se. P < 0.05 (compared to control) (paired t-test).
Results

Hemodynamics

Global hemodynamic parameters, such as heart rate, left ventricular pressure, and aortic pressure, remained unchanged over the entire experiment in the control-, ribose-, and AICAR-treated group (Table 1). In the adenosine-treated group, heart rate was significantly higher and aortic mean pressure lower during adenosine infusion, which is due to the peripheral vasodilating effect of adenosine. The hemodynamic changes were larger during the first hour of reperfusion and changed toward normalization during the following 2 hours of adenosine infusion, resulting in an 11% reduction of aortic mean pressure and a 10% increase of heart rate after 3 hours of adenosine infusion (Table 2). Subsequently, the instantaneous myocardial oxygen consumption (MVO₂) was found to be stable in a range between 6 and 7 ml O₂/min per 100 g in the control-, ribose-, and AICAR-treated group, but increased during adenosine infusion.

Effects of Ischemia on High-Energy Phosphates

In our experimental model, 45 minutes of regional ischemia led to a marked depression of tissue ATP by 50% (Fig. 1) and creatine phosphate by 80-90% (Table 3). AMP concentrations were doubled and ADP concentration remained unaltered. The breakdown products of AMP, like adenosine, inosine, and hypoxanthine/xanthine, were markedly increased. NAD was decreased by 24.6% (Table 3).

Effect of Reperfusion on High Energy Phosphates

ATP de Novo Synthesis

Adenine nucleotide de novo synthesis was measured by 1-[14C]glycine incorporation into nucleotides. Specific activity of [14C]glycine in myocardial tissue after 3 hours of [14C]glycine infusion is shown in Table 4. From the known specific activity and the detected radioactivity in the ATP and ADP, the nucleoside synthesis rates were calculated (Table 5). Basic ATP synthesis rate in nonischemic tissue was found to be very slow (3.74 nmol/g wet weight per

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Table 2

Heart Rate (HR) and Mean Aortic Pressure (AOP_m) after 45 Minutes of Ischemia and during 3 Hours of Reperfusion with Intracoronary Adenosine Infusion (3 mM/hr)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Ischemia (45 min)</th>
<th>Reflow (60 min)</th>
<th>Reflow (120 min)</th>
<th>Reflow (180 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR</td>
<td>99.5 ± 8.7</td>
<td>95.6 ± 7.1</td>
<td>132.1 ± 7.2*</td>
<td>123.2 ± 6.8†</td>
<td>113.4 ± 6.9†</td>
</tr>
<tr>
<td>AOP_m</td>
<td>113.0 ± 7.3</td>
<td>109.5 ± 5.7</td>
<td>83.4 ± 8.8*</td>
<td>92.6 ± 7.6†</td>
<td>99.0 ± 9.3†</td>
</tr>
</tbody>
</table>

* P < 0.02, † P < 0.05.

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Table 3

Nucleotides, Nucleosides, and Creatine Phosphate Levels after 45 Minutes of Ischemia and 3 Hours of Reperfusion with Different Substrate Infusion

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>45 Min of Ischemia</th>
<th>Saline</th>
<th>AICAR</th>
<th>Adenosine</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP (nm/g wet wt)</td>
<td>1120 ± 61</td>
<td>980 ± 82</td>
<td>822 ± 81*</td>
<td>912 ± 57*</td>
<td>748 ± 102*</td>
</tr>
<tr>
<td>AMP (nm/g wet wt)</td>
<td>68.1 ± 3.0</td>
<td>144.0 ± 9.0*</td>
<td>41.0 ± 3.1*</td>
<td>42.3 ± 2.8*</td>
<td>54.8 ± 17.1</td>
</tr>
<tr>
<td>IMP (nm/g wet wt)</td>
<td>7.4 ± 4.3</td>
<td>7.4 ± 5.1</td>
<td>ND</td>
<td>67.0 ± 10.0*</td>
<td>8.8 ± 4.3</td>
</tr>
<tr>
<td>Adenosine (nm/g wet wt)</td>
<td>9.0 ± 0.9</td>
<td>65.7 ± 15.4†</td>
<td>8.1 ± 0.9</td>
<td>7.3 ± 1.7</td>
<td>174.1 ± 36.8†</td>
</tr>
<tr>
<td>Inosine (nm/g wet wt)</td>
<td>9.2 ± 3.5</td>
<td>1131 ± 249†</td>
<td>5.2 ± 1.6</td>
<td>5.9 ± 1.7</td>
<td>212 ± 42.0†</td>
</tr>
<tr>
<td>Hypoxanthine (nm/g wet wt)</td>
<td>ND</td>
<td>261 ± 24.1</td>
<td>ND</td>
<td>ND</td>
<td>55.1 ± 9.9</td>
</tr>
<tr>
<td>AICAR (nm/g wet wt)</td>
<td>ND</td>
<td>ND</td>
<td>985 ± 266</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>AICAR-MP (nm/g wet wt)</td>
<td>ND</td>
<td>ND</td>
<td>572 ± 91</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>NAD (nm/g wet wt)</td>
<td>691 ± 83</td>
<td>522 ± 71*</td>
<td>543 ± 43*</td>
<td>580 ± 25*</td>
<td>498 ± 72*</td>
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<tr>
<td>CP (umol/g wet wt)</td>
<td>9.25 ± 0.73</td>
<td>2.75 ± 0.25†</td>
<td>12.26 ± 0.49*</td>
<td>12.94 ± 0.53*</td>
<td>10.52 ± 0.38</td>
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</table>

* P < 0.01; † P < 0.001 compared to control (paired t-test).
3 hr). Within the first 3 hours of reperfusion of previously ischemic tissue, the rate was doubled (7.48 nmol/g wet weight per 3 hr) (Table 5). The synthesis rates of adenine nucleotides were 3.07 nmol/g wet weight per hr in postischemic and 1.50 nmol/g wet weight per hr in nonischemic myocardium (Table 6). This slow synthesis rate would lead only to nonmeasurable increases of ATP within 3 hours of reperfusion. Therefore, the ATP levels in the control group with 45 minutes of ischemia and 3 hours of reperfusion remained depressed to 50% of normal (Fig. 1). AMP, adenosine, and inosine, and hypoxanthine levels returned to normal values. Creatine phosphate concentrations showed a marked increase compared to ischemic levels to a value of 15% above normal ('overshoot') (Table 3).

**Ribose as a Substrate for ATP Synthesis**

The synthesis rate of adenine nucleotides (AN) with ribose-infusion was determined in the same way as the AN de novo synthesis, by infusing ribose together with [14C]glycine. The synthesis rate was found to be 15.1 nmol/g wet weight per hr in postischemic and 9.8 nmol/g wet weight per hr in nonischemic tissue (Table 6). This results in a 5- to 6-fold increase of AN de novo synthesis. This increase is far too low to be measured in overall tissue ATP levels, and therefore no increase of ATP could be shown within 3 hours of reperfusion (Fig. 1). Creatine phosphate concentrations showed the same 'overshoot' phenomenon as in the saline-treated control group (control, 9.7 ± 0.6 µmol/g wet weight; 180 minutes of reperfusion, 12.2 ± 1.7 µmol/g wet weight).

**AICAR as a Substrate of ATP Synthesis**

AICAR was taken up by the myocardial cell and was phosphorylated to AICAR-monophosphate (AICAR-MP) (Fig. 2). At the same tissue AICAR concentration, the phosphorylation rate was higher in previously ischemic compared to nonischemic myocardium. Since AICAR concentrations were twice as high (previous ischemic areas) or 4-fold as high (nonischemic areas) as AICAR-MP concentration, phosphorylation seemed to be rate limiting. Areas that received AICAR only via blood recirculation in a 70-fold lower plasma concentration showed a 15-fold lower AICAR tissue concentration and a lower AICAR-MP concentration compared to infusion areas. This demonstrates a dose-dependent AICAR uptake and AICAR-MP formation (Fig. 2). Transformation of AICAR-MP to inosine monophosphate (IMP) is shown by the 9-fold increase of IMP in the tissue (Table 3) and the incorporation of 3H into IMP in [3H]AICAR-treated dogs (Fig. 3a; Table 7). This incorporation is dependent on the AICAR-MP levels. Only 15% of the AICAR-MP activity was found in IMP. A similar percentage was found in the increase of tissue levels of AICAR-MP and IMP. Further incorporation into the adenine nucleotide pool is shown by 3H incorporation into ADP and ATP (Table 7; Fig. 3, a and b). There are also linear

<table>
<thead>
<tr>
<th>Glycine Levels and Glycine Specific Activity after 3 Hours of 14C-Glycine Intracoronary Infusion in Postischemic and Nonischemic Myocardial Tissue</th>
<th>Postischemic</th>
<th>Nonischemic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine level (nmol/g wet wt)</td>
<td>815.2 ± 62.3</td>
<td>1041.5 ± 83.5</td>
</tr>
<tr>
<td>Specific activity (counts/min per nmol)</td>
<td>3830.2 ± 262.5</td>
<td>3974.3 ± 296.3</td>
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<table>
<thead>
<tr>
<th>Purine Nucleotide de Novo Synthesis and Accelerated Synthesis Rates after 3 Hours of Intracoronary Substrate Infusion in Previously Ischemic and Nonischemic Myocardium*</th>
<th>de Novo</th>
<th>Ribose</th>
<th>AICAR</th>
<th>Adenosine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Postischemic</td>
<td>3.07 ± 0.26</td>
<td>15.06 ± 0.39</td>
<td>27.4 ± 3.3</td>
<td>268.5 ± 45.2</td>
</tr>
<tr>
<td>Nonischemic</td>
<td>1.50 ± 0.31</td>
<td>9.77 ± 0.49</td>
<td>28.5 ± 4.2</td>
<td>218.7 ± 36.0</td>
</tr>
</tbody>
</table>

* In nmol/g wet weight per hour.
Mauser et al. / Acceleration of ATP Synthesis

FIGURE 1. Tissue ATP levels before ischemia (C), after 45 minutes of regional ischemia (I), and after 3 hours of reperfusion (R) with intracoronary infusion of ribose, AICAR, or adenosine. A group with saline infusion served as a control. Within 3 hours, only adenosine infusion leads to a significant increase in tissue ATP levels.

correlations between the [3H]AICAR-MP levels and radiolabel incorporation into ADP and ATP (Fig. 3, a and b). The net ATP synthesis can be calculated from the [3H] incorporation into ATP and from the known specific activity of infused AICAR. It results in a rate of ATP synthesis of 19 nmol/g wet weight per hr in areas of intracoronary AICAR infusion (Table 7). This increase is too small to be detected in overall tissue ATP-levels after 3 hours of reperfusion. Consequently, there was no difference between the endischemic ATP values and those, after 3 hours of reperfusion with AICAR (Fig. 1). A marked percentage of the AICAR-MP activity was found in AICAR triphosphate (AICAR-TP) which is synthesized in preference to ATP with high AICAR concentrations (Fig. 3b). Tissue AICAR-TP concentrations calculated from these data ranged between 0.15 and 0.3 μmol/g wet weight in the areas with intracoronary AICAR infusion. Inosine and hypoxanthine tissue levels stayed within normal limits during AICAR infusion (Table 3). Creatine phosphate showed the same postischemic overshoot as the control group.

Adenosine as a Substrate of ATP Synthesis

After [3H]adenosine infusion, radioactivity was found in AMP, ADP, and in large amounts in ATP, resulting in an adenine nucleotide synthesis rate of 268 nmol/g wet weight per hr (Table 7). A similar synthesis rate (323 nmol/g wet weight per hr) was calculated from the directly measured increase in tissue ATP concentrations (Fig. 1). Tissue adenosine levels were higher during adenosine infusion compared to values at the end of ischemia, demonstrating that the infused adenosine is only partially deaminated until it reaches the reperfused postischemic myocardium (Table 3).

Catabolism of AICAR and Adenosine

With AICAR infusion, no rise in tissue inosine or hypoxanthine concentrations was found (Table 3). Plasma uric acid concentrations increased with time with no difference between left atrial and vena cava inferior blood (Fig. 4a). A marked difference in AICAR levels between caval and left atrial blood but no difference in uric acid levels indicates peripheral uptake of AICAR and renal elimination of nonmetabolized AICAR (Fig. 4b).

Adenosine infusion is followed by high tissue inosine and hypoxanthine (Table 3) and high hypoxanthine levels in plasma (control, 0.18 ± 0.01 mg/liter; 3 hours of adenosine infusion, 1.21 ± 0.49 mg/liter), showing fast deamination of adenosine in blood and tissue. No adenosine was found in vena cava inferior blood. Uric acid levels were twice as high as after AICAR infusion (11.32 ± 0.25 mg/liter).

FIGURE 2. AICAR phosphorylation is shown by plotting the content of AICAR vs. AICAR monophosphate of single biopsies obtained from different sites of the heart. Comparison of ischemic (closed circles) and nonischemic (open circles) intracoronary infusion areas demonstrates a higher phosphorylation rate in previously ischemic areas, and comparison of infusion areas to areas that received AICAR via recirculation shows a dose-dependent build-up of AICAR monophosphate.
TABLE 7
Incorporation of [3H]AICAR or Adenosine Into Nucleotides* and Calculated Nucleotide Synthesis Rates†

<table>
<thead>
<tr>
<th></th>
<th>[3H]AICAR</th>
<th>[3H]Adenosine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Radioactivity (counts/min per g wet wt x 10^3)</td>
<td>Synthesis rate (nmol/g wet wt per 3 hr)</td>
</tr>
<tr>
<td>IMP</td>
<td>I</td>
<td>16.4 ± 4.5</td>
</tr>
<tr>
<td></td>
<td>NI</td>
<td>16.2 ± 6.2</td>
</tr>
<tr>
<td>AMP</td>
<td>I</td>
<td>11.2 ± 2.9</td>
</tr>
<tr>
<td></td>
<td>NI</td>
<td>11.2 ± 5.2</td>
</tr>
<tr>
<td>ADP</td>
<td>I</td>
<td>24.8 ± 4.9</td>
</tr>
<tr>
<td></td>
<td>NI</td>
<td>25.5 ± 5.4</td>
</tr>
<tr>
<td>AICAR-TP</td>
<td>I</td>
<td>81.8 ± 11.9</td>
</tr>
<tr>
<td></td>
<td>NI</td>
<td>80.1 ± 13.4</td>
</tr>
</tbody>
</table>

I = previously ischemic myocardium; NI = nonischemic myocardium.
* Within 3 hours of reperfusion.
† Specific activity of infused compounds: AICAR, 439.8 counts/min per nmol; adenosine, 451.9 counts/min per nmol.

Discussion

The slow recovery of the depleted adenine nucleotide pool, especially of ATP, following a period of potentially reversible ischemia is well recognized (DeBoer et al., 1980; Reimer et al., 1981; Swain et al., 1982b). Since the fall of ATP during ischemia is not accompanied by a rise in ADP, we must conclude that ADP is quickly transformed to AMP, which is found to be doubled after ischemia in our experiments. The myokinase reaction and dephosphorylation of AMP by 5’-nucleotidase leads to adenosine which is deaminated to inosine. Both nucleosides can leave the cell. During reflow, the nucleotide degradation products are washed out of the interstitial space (Katori and Berne, 1966; Fox et al., 1979) and are unavailable for the nucleotide salvage pathways (Maguire et al., 1972; Parker et al., 1976). The cell is now dependent on the very slow and energy-consuming de novo synthesis. Our experiments and those of Zimmer et al. (1973) showed a doubling of the AN de novo synthesis during recovery from oxygen deprivation, but, in our dog experiments, the de novo synthesis rate was only 38% (3 nmol/g wet weight per hr) compared to the rate measured in rats. Therefore, it would have taken about 14 days to synthesize 1.0 μmol/g wet weight of ATP. There are two reasons for this. First, ribose-5-phosphate, the basic substrate of AN de novo synthesis was shown to be in short supply after ischemia (Zimmer et al., 1973). Second, the energy-consuming assembly of the purine structure by successive attachment of C-fragments to phosphoribosylpyrophosphate is slow in energy-depleted cells. It was already shown by Zimmer (1980) that exogenous supply of ribose can speed up ATP synthesis 3- to 4-fold in the rat heart. In our dog model, ribose infusion leads to similar results by accelerating AN de novo synthesis 4- to 6-fold. This acceleration is far too slow to achieve a measurable increase of ATP within 3 hours of reperfusion. These results also show that the acceleration of AN synthesis by AICAR or adenosine cannot be due to a splitting of the molecule and incorporation of the ribose moiety of the molecule, because acceleration rates of AN synthesis are higher with AICAR and adenosine, as compared to ribose.

Studies with AICAR

A new approach bypassing several steps of the AN de novo synthesis pathway was the infusion of AICA-riboside (AICAR) as shown by Sabina et al. (1982). AICAR is the dephosphorylated form of the AN de novo synthesis intermediate, AICAR monophosphate (AICAR-MP). It enters the biosynthetic pathway distal to the major control points (Wyngaarden and Ashton, 1959) and needs only one molecule of ATP and GTP to form AMP. Swain et al. (1982a) showed a 25% increase of ATP (from a postischemic 70-95% of normal) within 24 hours of AICAR infusion in the dog. In our study, with a more severe ischemia and a reduction of ATP to 50% of normal, we demonstrated incorporation of tritium-labeled AICAR into ATP representing a 9-fold increase of AN synthesis within the first 3 hours of reperfusion compared to the de novo synthesis that was measured by [14C]glycine incorporation and a 20-fold increase of AN synthesis in nonischemic tissue. This acceleration is twice that for ribose, but is still too small to be detected from overall tissue ATP levels within 3 hours of reperfusion. There are two reasons for the still insufficient acceleration of ATP synthesis by AICAR. First, uptake, especially in postischemic tissue, and phosphorylation of AICAR to AICAR monophosphate by myocytes, is slow: AICAR con-
centrations are twice as high as AICAR-MP concentrations in previously ischemic tissue, and four times higher in nonischemic tissue. Second, with higher AICAR monophosphate concentrations, the preferred metabolic pathway is the transformation of AICAR-MP to AICAR triphosphate. AICAR triphosphate formation from AICAR has also been shown by Zimmerman and Deeprose (1978) in erythrocytes, by Bochner et al. (1982) in cells after 10-tetrahydrofolate deficiency, and in small amounts by Sabina et al. (1982) in the heart after intraatrial AICAR infusion. The AICAR triphosphate levels reported by Sabina are in the same range as the levels we found in myocardial areas that received AICAR via recirculation. The AICAR triphosphate formation and ATP buildup is equal. With higher AICAR-MP levels in areas that received AICAR via intracoronary infusion, AICAR triphosphate formation becomes predominant and leads to AICAR triphosphate levels of 0.15–0.3 μmol/g wet weight within 3 hours of reperfusion. A reported inhibition of the enzyme adenylosuccinate lyase by AICAR-MP (Sabina et al., 1982), which catalyses the transformation of IMP to AMP, could not be observed in our study, since there was a linear correlation between AICAR concentrations and ATP built up over a 20-fold range encompassing myocardial areas that received AICAR only via recirculation and myocardial areas that received AICAR via intracoronary infusion. From our data, we cannot decide whether the metabolism of AICAR to ATP is linear during the 3-hour infusion period. However, the constant increase of uric acid in plasma shows a linear catabolism of infused AICAR. The slow time-dependent increase of uric acid in plasma with no differences between left atrial and
Figures 4. Panel a: AICAR infusion leads to a slow time-dependent increase of plasma uric acid levels with no statistical difference in left atrial and vena cava inferior levels. After stopping AICAR infusion, uric acid is slowly eliminated. (Statistics: 10 minutes and 30 minutes of AICAR infusion, \( P < 0.002 \) compared to control; 60-180 minutes of AICAR infusion, \( P < 0.001 \) compared to control.) Panel b: with intracoronary AICAR infusion, plasma AICAR levels rise within the first hour of infusion. A marked difference between left atrial and vena cava inferior AICAR concentrations under steady state conditions with no difference in uric acid levels suggests peripheral uptake and kidney elimination of unmetabolized AICAR. Stopping AICAR infusion leads to a sharp fall of plasma AICAR concentrations.

Venous concentrations over the whole infusion period is indicative of a slow catabolism of AICAR. The very high plasma levels of AICAR are also a parameter for the slow metabolism, and the great difference between left atrial plasma levels and vena cava inferior plasma levels under steady state conditions after 1 hour of AICAR infusion can be explained by uptake in peripheral tissue and renal excretion of nonmetabolized AICAR. The time-dependent increase of plasma uric acid concentration after AICAR infusion would suggest IMP formation. Since IMP is a crossroad of three metabolic pathways (IMP to AMP, IMP to GTP, IMP to inosine), we are not surprised to find a much lower radiolabel incorporation into ATP as compared to adenosine: most of the newly formed IMP is probably degraded to uric acid. The complete absence of a rise in inosine and hypoxanthine above normal levels in tissue and plasma with AICAR infusions is, however, difficult to explain, and would suggest the possibility of a route from AICAR to uric acid not encompassing IMP. It also shows that the AICAR dose used is appropriate for investigation of the effects of AICAR. A further increase of AICAR-MP concentration with higher AICAR concentrations would lead only to a minimal increase in ATP formation. A decrease in AICAR concentration leads to a decrease in ATP formation as shown.

Studies with Adenosine

An increase of ATP in postischemic myocardium is reported with adenosine as a precursor (Reibet and Rovetto, 1979; Isselhard et al., 1980; Foker et al., 1980). Since most of these studies have been done in isolated rat or guinea pig hearts, no addition of an inhibitor of adenosine deaminase for preventing adenosine deamination in blood (van Belle 1969) was necessary. In a whole animal preparation, acceleration of ATP synthesis was successful only when intravenous adenosine was given together with EHNA (erythro-g-(2-hydroxy-3-nonyl)-adenine, a potent adenosine deaminase blocker. As far as we know, adenosine was therefore not used to replenish decreased ATP pools rapidly in regionally ischemic and reperfused myocardium in the dog heart. Foker's study (Foker et al., 1980) was done in
the totally and globally ischemic heart within the context of a cardioplegic study. Isselhard et al. (1970b, 1980) studied adenosine either in postasphyxic rabbit hearts or in normal dog hearts. The intracoronary administration of adenosine has the advantage of partially bypassing the adenosine deamination by blood which is correlated with contact time (van Belle, 1969). It leads only to minimal adenosine concentrations in the peripheral tissues after passing through the lungs, i.e., it does not recirculate. This allows high local adenosine concentrations with only minor peripheral hemodynamic changes. It cannot be ruled out that these hemodynamic changes may influence ATP catabolism; however, Isselhard (1980, 1984, in press) has shown recently that the hemodynamic changes produced by adenosine do not affect ATP synthesis. Since myocardial oxygen consumption increases slightly after adenosine infusion, due to an increase of heart rate, there is probably an increase in ATP turnover. This may lead to a small underestimation of accelerated ATP synthesis by adenosine. High tissue adenosine levels demonstrate the uptake of adenosine from the blood. Increased inosine and hypoxanthine tissue levels show that part of the adenosine is rapidly deaminated. The \( ^{3}H \) incorporation found in ATP after infusion of \( ^{3}H \)adenosine, as well as the increase of total ATP from 50% postischemic to 75% of normal within 3 hours of reperfusion results in a 90-fold increase of ATP synthesis compared to the de novo synthesis. These incorporation rates are comparable to those reported in studies with isolated perfused hearts (Reibel and Rovetto, 1979; Isselhard et al., 1980). Adenosine transformation to AMP is catalyzed by the enzyme adenosine kinase which is not an enzyme of the purine de novo synthesis pathway. Incorporation of both ribose and AICAR is dependent on the pre-existent purine de novo synthesis pathway. However, ribose enters this pathway after being converted to ribose-5-phosphate and phosphoribosylpyrophosphate, at the beginning, and AICAR enters after conversion to AICAR-MP, near the end, of this pathway.

There seemed to be several rate-limiting steps. One is the availability of phosphoribosylpyrophosphate which can be overcome by the infusion of ribose. However, this can speed up the synthesis only by a factor of five to six, which is minimal compared to adenosine incorporation. Because of the big difference in the incorporation rate of AICAR and adenosine, there must also be a rate-limiting step in the conversion of AICAR-MP to AMP. Since there is no increase of AICAR-MP after ribose infusion, the capacity of the conversion of ribose-5-phosphate to AICAR-MP seems also to be limited. The conversion of phosphoribosylpyrophosphate to IMP is dependent on ATP, which is needed at several steps of this pathway. This seems to be no problem, even for the ATP-depleted cell, because ribose incorporation is doubled in postischemic tissue with lower ATP levels and there is no big difference in the incorporation of ribose and AICAR, whereas AICAR enters this pathway distal to the ATP-dependent steps.

Each of the three tested substrates is able to restore postischemic reduced myocardial ATP levels significantly faster than ATP-de novo synthesis, but there are big differences in the extent of incorporation of these precursors into the adenine nucleotide pool. Adenosine is rapidly incorporated and leads to a fast increase of tissue ATP levels within hours.

Incorporation of ribose or AICAR is much slower, and a measurable increase of ATP levels in dog myocardium would only be expected after 1-2 days.

The ability of fast restoration of ATP levels leads to two questions that have to be resolved in further experiments, i.e., (1) will reduced contractile function improve with higher ATP levels and (2) will partially restored myocardial ATP levels protect the heart against recurrent episodes of ischemia?

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INDEX TERMS: Adenosine • AICAR • Ribose • ATP • Reperfusion
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