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J Clin Invest. 1994;**93**(1):40-49. <https://doi.org/10.1172/JCI116974>.

Research Article

Capacity for ATP resynthesis during recovery from ischemia or hypoxia is limited to the size of the adenine nucleotide pool, which is determined in part by the activity of cytosolic 5'-nucleotidase (5'-NT): AMP \rightarrow adenosine plus inorganic phosphate (Pi). To define in vivo regulation of 5'-NT, we used the tools of ³¹P nuclear magnetic resonance (NMR), spectroscopy and chemical assay to measure the substrates (AMP), products (Pi, adenosine, and its catabolites), and inhibitors (Pi and H⁺) of 5'-NT in isolated perfused rat hearts exposed to hypoxia (where pH remains near 7) and no flow, global ischemia (where pH falls to 6.1). We estimated 5'-NT reaction velocity, assessed the relative contributions of Pi and H⁺ to enzyme inhibition, and defined the consequences of changes in 5'-NT activity on ATP resynthesis after hypoxia and ischemia. We conclude that (a) 5'-NT is activated during hypoxia and early ischemia but is inhibited during prolonged ischemia, (b) H⁺ (pH < 6.2) is a potent inhibitor of 5'-NT, and (c) differences in AMP accumulation are sufficient to explain the differences in the capacity for net ATP resynthesis in ischemic and hypoxic tissue. These observations have implications for our understanding of heterogeneity of ischemic injury and myocardial protection during ischemia.

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Acidosis during Ischemia Promotes Adenosine Triphosphate Resynthesis in Postischemic Rat Heart

In Vivo Regulation of 5'-Nucleotidase

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Abstract

Capacity for ATP resynthesis during recovery from ischemia or hypoxia is limited to the size of the adenine nucleotide pool, which is determined in part by the activity of cytosolic 5'-nucleotidase (5'-NT): AMP → adenosine plus inorganic phosphate (Pi). To define in vivo regulation of 5'-NT, we used the tools of ³¹P nuclear magnetic resonance (NMR) spectroscopy and chemical assay to measure the substrates (AMP), products (Pi, adenosine, and its catabolites), and inhibitors (Pi and H⁺) of 5'-NT in isolated perfused rat hearts exposed to hypoxia (where pH remains near 7) and no flow, global ischemia (where pH falls to 6.1). We estimated 5'-NT reaction velocity, assessed the relative contributions of Pi and H⁺ to enzyme inhibition, and defined the consequences of changes in 5'-NT activity on ATP resynthesis after hypoxia and ischemia. We conclude that (a) 5'-NT is activated during hypoxia and early ischemia but is inhibited during prolonged ischemia, (b) H⁺ (pH < 6.2) is a potent inhibitor of 5'-NT, and (c) differences in AMP accumulation are sufficient to explain the differences in the capacity for net ATP resynthesis in ischemic and hypoxic tissue. These observations have implications for our understanding of heterogeneity of ischemic injury and myocardial protection during ischemia. (*J. Clin. Invest.* 1994. 93:40-49.)
Key words: Adenosine triphosphate resynthesis • ³¹P nuclear magnetic resonance spectroscopy • hypoxia • ischemia • reperfusion

Introduction

Acute myocardial infarction, recurrent myocardial ischemia, and cardiac protection during cardiopulmonary bypass all require clinical management designed to reestablish coronary blood flow and to minimize the size of ischemic damage. Although many metabolic processes are involved in reperfusion injury, it is well established that ischemia leads to a loss of ATP content and that upon reperfusion there is incomplete resynthesis of ATP. Since the availability of cytosolic ATP is essential for myocardial contraction and relaxation, it has been postulated that prolonged depletion of ATP could contribute to me-

chanical dysfunction of the myocardium during reperfusion (1-3). Consequently many experimental studies have focused attention on ways to increase ATP content during reperfusion. These include supplying pharmacological agents during preischemia to slow the rate of ATP hydrolysis during ischemia (4), expanding the size of the purine pool in an attempt to slow the loss of purines or to stimulate salvage pathways leading to ATP synthesis (5, 6), and preconditioning (7). However, few studies have attempted to define the mechanisms responsible for ATP resynthesis after ischemia.

In myocardial cells injured by ischemia or hypoxia, ATP is degraded to nucleotides, nucleosides, and nucleobases via the following pathway: ATP → ADP → AMP → adenosine → inosine → hypoxanthine → xanthine. The nucleotides ATP, ADP, and AMP do not cross the cell membrane, but the nucleosides and nucleobases adenosine, inosine, and hypoxanthine diffuse across the cell wall down their concentration gradients. The presence of coronary flow exacerbates the loss of purines. In this way the purine pool is depleted during hypoxia and low flow ischemia, and during early reperfusion after total ischemia. The ability to resynthesize ATP during the first few minutes of recovery from ischemia is limited by the size of the remaining endogenous pool of adenine nucleotides. The larger the adenine nucleotide pool during ischemia, the greater the capacity for ATP resynthesis upon reperfusion. Thus, the conversion of AMP to adenosine is a critical step in this process.

Cytosolic AMP is metabolized by the action of 5'-nucleotidase (5'-NT¹, E.C. 3.1.3.5) (AMP → adenosine plus inorganic phosphate [Pi] (8-10) and AMP deaminase (AMP → IMP plus NH₄⁺) (11). Recent results using human (12) and animal (11) myocardium show that the AMP deaminase reaction makes a relatively minor contribution and that 5'-NT is the predominant reaction for AMP degradation in the heart.

Studies of cytosolic 5'-NT purified from mammalian and avian hearts have demonstrated that 5'-AMP is its primary substrate (8-10). Results from recent in vitro studies also provide evidence that activity of AMP-specific cytosolic 5'-NT is inhibited by high concentrations of Pi and protons (H⁺) (8, 9). If this is also the case in vivo, then inhibition of 5'-NT by acidosis would lead to an increase in AMP content during ischemia when [H⁺] is high, but not during hypoxia when [H⁺] is increased only slightly, and ATP resynthesis from AMP would occur during reperfusion but not during reoxygenation. In this way, acidosis would protect the severely ischemic myocardium from further depletion of adenine nucleotides, thereby setting the stage for rapid resynthesis of ATP upon reperfusion.

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Received for publication 29 January 1993 and in revised form 1 June 1993.

J. Clin. Invest.

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0021-9738/94/01/0040/10 \$2.00

Volume 93, January 1994, 40-49

1. Abbreviations used in this paper: NMR, nuclear magnetic resonance; 5'-NT, 5'-nucleotidase; PCr, phosphocreatine; Pi, inorganic phosphate; PME, monophosphate ester.

To test this hypothesis, we took advantage of the fact that in isolated perfused small animal hearts, ATP is depleted during both hypoxia and ischemia, but the extent of H^+ accumulation differs. During global ischemia, intracellular pH (pH_i) declines from normal values of 7.1 to values as low as 6.1 (13) but during high flow hypoxia pH_i remains close to 7.0 (14). Accordingly, we compared rates of ATP depletion and AMP and H^+ accumulation during hypoxia and ischemia and in subsequent reoxygenation and reperfusion periods in isolated rat hearts. By using ^{31}P nuclear magnetic resonance (NMR) spectroscopy we measured not only ATP content and pH_i , but also phosphocreatine (PCr) and Pi contents. Combining these values with the measurement of total creatine, we calculated cytosolic ADP and AMP concentrations using equilibrium expressions for the creatine kinase and adenylate kinase reactions. We also compared these calculated values of ADP and AMP concentrations to those measured in the intact heart using ^{31}P NMR spectroscopy and chemical assay. Finally, the concentrations of the diffusible adenine nucleosides and nucleobases were also measured using chemical assays. In this way we measured all known substrates, products, and regulators of 5'-NT activity in these in vivo preparations.

Methods

Rat heart preparation. Experiments were carried out on hearts of male Sprague-Dawley rats (Charles River Breeding Laboratories, Inc., Kingston, CT) weighing between 375 and 450 g. Anesthesia was achieved by intraperitoneal injection of 20 mg sodium pentobarbital. After performing a transverse laparotomy and an anterolateral thoracotomy, the beating heart was removed rapidly and placed into cold Krebs-Henseleit buffer. The heart was attached to a perfusion apparatus via the aorta and perfused in the isovolumic, Langendorff mode at a constant temperature of 37°C and a constant perfusion pressure of 100 mmHg. The perfusate was phosphate-free Krebs-Henseleit buffer of the following composition (mM): NaCl 118, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 1.75, EDTA 0.5, glucose 11, $NaHCO_3$ 25, and bubbled with 95% O_2 and 5% CO_2 , giving a pH of 7.4. A small polyethylene tube was placed through the apex to drain flow from the Thebesian veins. A water-filled balloon was inserted into the left ventricle through the mitral valve and connected to a pressure transducer (Statham P23Db; Gould Instruments, Oxnard, CA) for continuous measurement of heart function. Heart performance was estimated as the product of heart rate and developed pressure. Coronary flow was determined by collecting coronary sinus effluent; control coronary flow was 16.7 ± 1.7 ml/min⁻¹ per g wet wt⁻¹. The heart was then immersed in its effluent inside a glass NMR tube.

Experimental protocols. Each heart underwent a stabilization period of about 20 min. Baseline data defining cardiac performance, pH_i , and high energy phosphate content were then collected. In a preliminary set of experiments, stability of hearts was assessed during 90 min of control perfusion. There was < 10% change per hour in mechanical (performance and coronary flow) and metabolic (from ^{31}P NMR spectra) parameters. For experimental hearts, baseline data were collected every 4 min during a 28-min period of control perfusion. Hearts were then divided into one of three protocols. ^{31}P NMR spectra were obtained throughout each protocol, with one spectrum obtained every 4 min.

In the first protocol, 10 hearts were continuously perfused for an additional 28 min under normoxic conditions (total time 56 min) and served as controls.

In the second protocol, hearts were subjected to either 12 ($n = 3$) or 28 min ($n = 8$) of global, normothermic, (37°C) no-flow ischemia. Ischemia was induced by occlusion of the cannula connected to the aorta. In two other groups of hearts, the ischemic periods were followed

by reperfusion for 28 min ($n = 3$ and $n = 6$ for reperfusion after 12 and 28 min of ischemia, respectively).

In the third protocol, two different groups of hearts were exposed to 28 min of hypoxia, produced by equilibration of buffer with 95% N_2 and 5% CO_2 ($pO_2 \sim 20$ Torr). In one of these groups, the protocol was terminated at the end of hypoxia ($n = 9$); in the other, the hypoxic period was followed by reoxygenation for 28 min ($n = 8$).

At the end of the experiment, each heart was freeze-clamped with aluminum tongs precooled in liquid nitrogen and was stored at -80°C until purine and protein contents were assayed.

NMR measurements. NMR measurements were made using an NMR spectrometer (NT 360; Nicolet Magnetics Corp., Fremont, CA). The perfused heart was placed in a 20-mm glass tube (Wilma Glass Co., Buena, NJ) and inserted into a $^{23}Na/^{31}P$ double-tuned probe which was seated in a wide-bore (89-mm) superconducting 8.4 magnet (Tesla; Oxford Instruments, Oxford, UK). Field homogeneity was optimized on the ^{23}Na signal in the sample (line width was 9–18 Hz) with the aid of an 18-channel Shim Supply (Oxford Instruments). For ^{31}P NMR spectroscopy (frequency 145.75 MHz) the pulse angle used was 45° (23 μ s pulse time) using a sweep width of $\pm 3,000$ Hz and 2,000 data points. At the beginning of each study, a fully relaxed (10 s repetition time) spectrum was acquired. Subsequently, partially saturated ^{31}P NMR spectra (2.18 s interpulse delay) were obtained, averaging data from 104 free induction decays (4 min total time). Comparing fully relaxed and partially saturated spectra showed that the nucleotide triphosphate resonances were fully relaxed ($\pm 5\%$) for all conditions; for PCr, Pi, and monophosphate esters (PME) resonance areas correction factors for saturation were required (1.2, 1.15, and 1.15, respectively). Spectra were analyzed using exponential multiplication of 20 Hz and phasing with zero and first-order phase correction. Peak areas were fitted by Lorentzian line shapes by using the software program (NMR1, Syracuse, NY).

Chemical assay of metabolites. Frozen ventricular tissue (10–20 mg wet wt) from each heart was pulverized in a stainless steel percussion mortar under liquid nitrogen and extracted by using 0.6 N perchloric acid. A portion of this homogenate was taken for protein assay by the method of Lowry et al. (15). After neutralization and centrifugation, diadenosine pentaphosphate (100 μ mol/ml⁻¹), an inhibitor of adenylate kinase, was added. One aliquot of the supernatant (200 μ l) was analyzed by high pressure liquid chromatography using a SAX column (Partisil 10; Whatman Inc., Clifton, NJ). Nucleotides (ATP, ADP, IMP) were eluted at a flow rate of 1.5 ml/min⁻¹ using a concave gradient of 0.007 M KH_2PO_4 buffer (pH 4.0) to 0.25 M KH_2PO_4 buffer (pH 4.5). A second aliquot (150 μ l), used for measurements of AMP, adenosine, inosine, hypoxanthine, and xanthine, was injected into a reverse-phase C_{18} column (μ Bondapak; Whatman Inc.) with 0.025 M $NH_4H_2PO_4$ buffer (pH 4.3); the flow rate was 2 ml/min⁻¹. The peaks were identified by comparison with retention times of known external standards and quantified by peak area. The amount of each adenine-containing compound was determined by absorbance at 254 nm using appropriate extinction coefficients. Note that since uric acid is present in only low concentrations in normal or ischemic myocardium (16), it was not measured in these studies. In other experiments, we used a third aliquot (70 μ l) for measurement of creatine using the method of Kammermeier (17). Results are expressed as nmol/mg protein⁻¹. All chemicals were obtained from Sigma Chemical Co., St. Louis, MO.

Data analysis. Cytosolic NTP concentrations measured by NMR spectroscopy in control hearts were calculated from the [β -P] resonance area by using results from an external ATP standard and knowing the NMR-sensitive volume and measured values of heart weight and protein content (18). The area of the [β -P] NTP of the spectra obtained under control perfusion was set to 100% and used as the reference value for all resonances in all spectra. In three additional hearts perfused for 16 min at a constant pressure and temperature, the ATP concentration determined by chemical assay was 33.4 ± 3.2 nmol/mg protein⁻¹. Using a measured value of 0.155 mg per milligram blotted wet weight for protein and the literature value of 0.48 μ l for the volume of intracellular water per milligram wet weight (19), this value

corresponds to a cytosolic ATP concentration of 10.8 mM. This value was taken as the internal standard to calculate time-dependent changes in the intracellular concentrations of PCr, Pi, and ATP in all experiments.

Cytosolic ADP and AMP concentrations were calculated using creatine kinase and adenylate kinase equilibrium expressions (20), with values determined from ^{31}P NMR and biochemical assays:

$$\text{ADP} = \frac{[\text{ATP}] \times [\text{creatine}]}{[\text{PCr}] \times [\text{H}^+] \times K_{\text{CK}}}, \text{ where } K_{\text{CK}} = 1.66 \times 10^9 \text{ M}^{-1}$$

$$\text{AMP} = K_{\text{MK}} \frac{[\text{ADP}]^2}{[\text{ATP}]}, \text{ where } K_{\text{MK}} = 1.05$$

Intracellular pH was measured by comparing the chemical shift between inorganic phosphate and creatine phosphate with values obtained from a standard curve.

Statistical analysis. All data are presented as mean values \pm one standard deviation. The statistical significance of the results was determined by using repeated measures analysis of variance followed by a Student's paired *t* test to determine differences between groups by using a computer program (Statview TM 512+; Brainpower, Inc., Calabasas, CA). A value of *P* < 0.05 was considered significant.

Results

Effects of myocardial hypoxia and ischemia on ATP, PCr, and Pi contents: ^{31}P NMR measurements

Representative sets of ^{31}P NMR spectra obtained for hearts exposed to either 28 min of hypoxic perfusion (A) or 28 min of global, normothermic ischemia (B) are shown in Fig. 1. The first spectrum in each set was obtained immediately before imposing either hypoxia or ischemia and the final spectrum was obtained at the end of the period of either reoxygenation or reperfusion; intervening spectra were obtained at 4-min intervals during hypoxia or ischemia. Fig. 2 shows the time course of the mean values for cytosolic ATP, PCr, and Pi contents during these two experimental protocols for all hearts studied.

During 28 min of hypoxia, the ATP content slowly declined ($\sim 0.17 \text{ mM}/\text{min}^{-1}$) to $58 \pm 11\%$ of prehypoxic values. The rate of PCr depletion was much faster. By 8 min of hypoxia, PCr content was $45 \pm 13\%$ of prehypoxic values. Thereafter PCr slowly declined to $27 \pm 5\%$ at the end of 28 min of hypoxia. The Pi content increased rapidly and by 12 min reached its maximum value of four times the prehypoxic value. Upon reoxygenation, the ATP content neither increased nor decreased significantly and remained at about 64% of prehypoxic values throughout the 28 min of reoxygenation (*P* < 0.001 compared with prehypoxic values). Unlike ATP, both PCr and Pi showed complete recovery to prehypoxic levels.

The rate and extent of ATP depletion was much faster during global, normothermic, no-flow ischemia ($\sim 0.42 \text{ mM}/\text{min}^{-1}$) than during an equivalent duration of hypoxia. After 20 min of ischemia, the concentration of ATP decreased to 9% of preischemic values. After 24 min of ischemia the $[\beta\text{-P}]$ peak of each ^{31}P NMR spectrum was no longer well resolved and therefore ATP was not detectable by ^{31}P NMR (< 0.3 mM). PCr content was also more rapidly depleted during ischemia than during hypoxia. After 4 min of ischemia PCr was only 22% of preischemic values and after 8 min the PCr peak was no longer detectable. The Pi levels rose rapidly and continuously, reaching values 5-fold and 10-fold higher than during preischemia by 12 and 28 min of ischemia, respectively. Because ATP and PCr contents were totally depleted, Pi levels are much higher during ischemia than during hypoxia. Restoration of

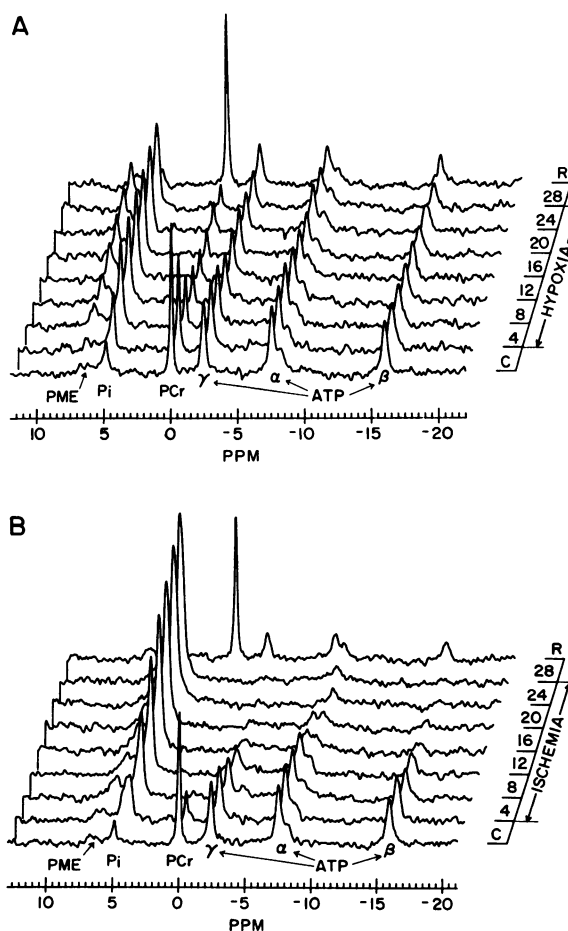


Figure 1. Representative stacks of ^{31}P NMR spectra from a rat heart subjected to either hypoxic (A) or ischemic (B) injury. Each spectrum represents 104 acquisitions obtained over a period of 4 min using a pulse angle of 45° and a recycle time of 2.18 s. The duration (min) of hypoxia or ischemia is shown to the right of each panel. The first (C) and last (R) are spectra obtained from heart after 28 min of oxygenated perfusion and at the end of 30 min of reoxygenation after a period of experimental injury.

perfusion after 28 min of ischemia caused rapid, but incomplete, recovery of ATP from nondetectable levels to $42 \pm 3\%$ of preischemic values. ATP content remained constant throughout the remaining 24-min period of reperfusion. Like the complete return of PCr and Pi after hypoxia, upon reperfusion after ischemia the PCr content rose rapidly and within 4 min was equal to or greater than preischemic values. Similarly, Pi rapidly returned to baseline values.

These results show that in spite of nearly complete depletion of ATP during ischemia, there was significant ATP resynthesis upon reperfusion. In contrast, in reoxygenated myocardium after hypoxic insult there was no net resynthesis of ATP. This is the case even for longer periods of hypoxic perfusion (not shown). Complete restoration of myocardial PCr content after both ischemia and hypoxia shows that myocytes remained metabolically intact.

Effects of myocardial hypoxia and ischemia on pH_i : ^{31}P NMR measurements

The chemical shift of the Pi resonance, but not the PCr resonance, is pH dependent and moves upfield as the cytosol be-

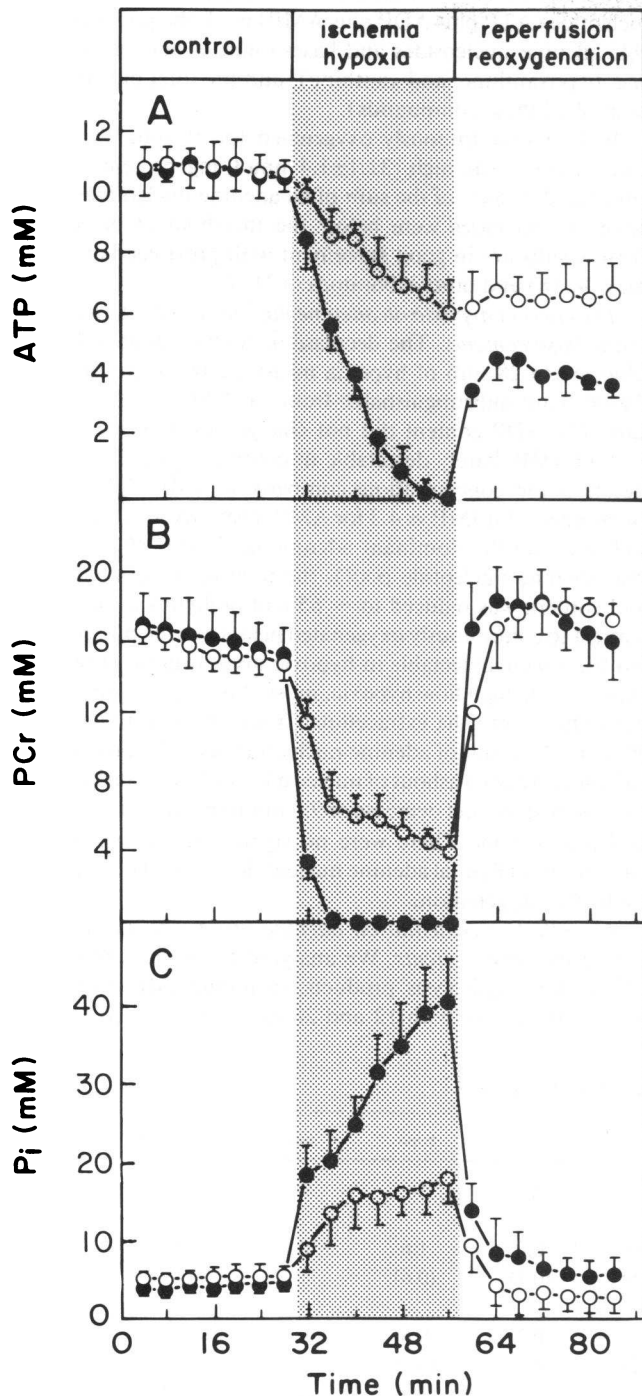


Figure 2. Changes in ATP (A), PCr (B), and Pi (C) concentrations (mM) throughout experimental protocols in rat hearts exposed to hypoxia (○) or ischemia (●). Data represent mean values \pm SD.

comes acidotic. Fig. 3 shows the region of ^{31}P NMR spectra containing the PCr and Pi resonances (9 to -1 parts per million [ppm]) from representative hearts at the end of control, hypoxia, reoxygenation, ischemia, and reperfusion periods. Fig. 4 depicts the time course of mean pH_i values during hypoxia and reoxygenation, and during ischemia and reperfusion.

Throughout 56 min of continuous oxygenated perfusion, the pH_i remained the same, 7.11 ± 0.02 vs 7.12 ± 0.02 at the beginning and end of perfusion, respectively (intervening data

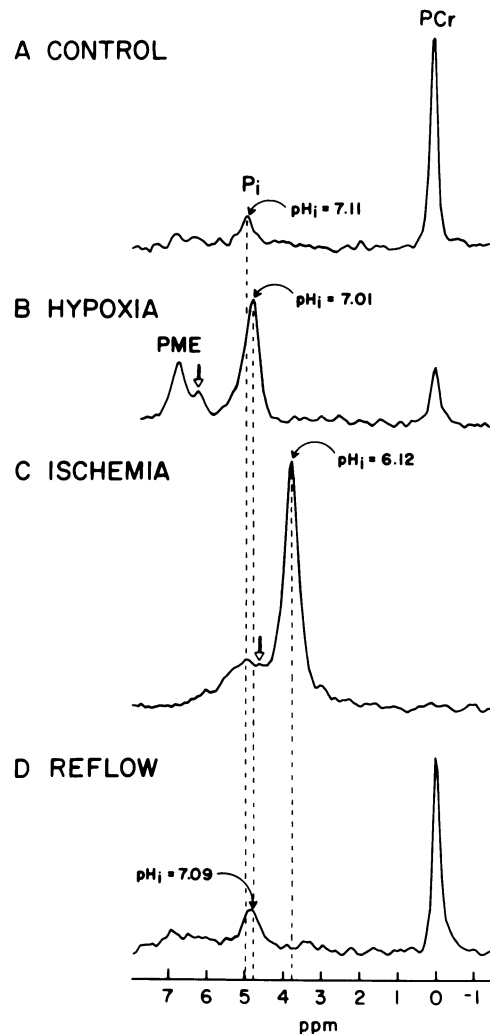


Figure 3. The expanded ^{31}P NMR spectra that were obtained from rat hearts subjected to 56 min of normoxic perfusion (A) or 28 min of either hypoxia (B) or ischemia (C) or at the end of reperfusion period (D). Open arrows point to the appropriate resonance position for AMP (see Results).

not shown). During hypoxia, pH_i declined only slightly. Although the change was small, to 7.00 ± 0.06 , it was significant ($P < 0.05$). With reoxygenation, pH_i recovered to prehypoxic levels within 8 min. In contrast, in ischemic hearts pH_i decreased rapidly ($0.09 \text{ pH unit}/\text{min}^{-1}$) from 7.10 ± 0.02 to 6.39 ± 0.08 by 8 min after cessation of flow ($P < 0.001$). After 12 min of ischemia, the pH_i fell to 6.17 ± 0.12 and remained at this low value until the end of the 28-min ischemic period (6.12 ± 0.12). With restoration of flow after ischemia, pH_i returned rapidly to the preischemic values and remained near $7.08 (\pm 0.03)$ throughout the reperfusion period. Since we obtained ^{31}P NMR spectra by signal averaging over 4 min, pH_i represents the average pH_i over the entire time interval. Hence we may conclude that the return of pH_i to control values after ischemia was very rapid.

These results show that intracellular pH changes very little during high flow hypoxia but decreases by about 1 pH U after 12 min of global, no flow ischemia in isolated buffer-perfused rat hearts.

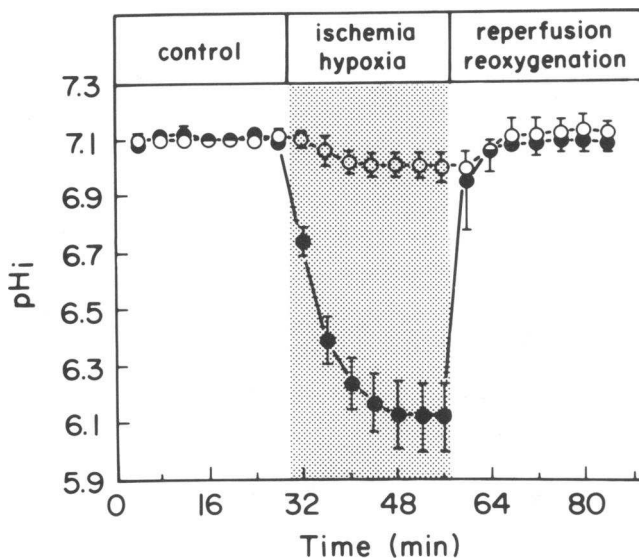


Figure 4. Changes in pH_i in hearts exposed to 28 min of hypoxia or 28 min of ischemia, followed by a 28-min period of reoxygenation or reperfusion. pH_i did not change during 56 min of normoxic perfusion (not shown).

Chemical analysis of ATP hydrolysis products in control, hypoxic, and ischemic myocardium (Table I)

The ^{31}P NMR experiments provide little or no information about the accumulation of purine-containing hydrolysis products of ATP during and after hypoxic and ischemic insults. This is because the concentrations of cytosolic ADP and AMP usually do not reach NMR-detectable levels or are difficult to resolve and because nonphosphorylated ATP hydrolysis products are not observable. To analyze the ATP hydrolysis products, we measured these compounds using chemical assay of acid extracts prepared from the same hearts used for ^{31}P NMR

studies. In this way we were able to measure the total adenine nucleotide (ATP plus ADP plus AMP) pool, the pool of diffusible adenine nucleosides and bases (sum of adenosine, inosine, hypoxanthine, and xanthine), and the total purine pool (sum of all these compounds).

In hearts continuously oxygenated for 56 min, the ATP concentration was high, 31.1 ± 1.4 nmol/mg protein $^{-1}$, and constituted $\sim 84\%$ of the sum of all adenine nucleotides. Nucleosides and bases were below the threshold of detection. These results are in good agreement with previous data from this (14, 18) and other laboratories (21–23).

The effect of hypoxia on adenine nucleotide, nucleoside, and purine base contents. The decrease in ATP content in hearts subjected to 28 min of hypoxia measured by chemical assay ($50 \pm 9\%$) are indistinguishable from ^{31}P NMR ($58 \pm 11\%$) results. The ADP content did not change but AMP increased 2.7-fold. IMP, barely detectable in control myocardium, was present in low concentrations compared to AMP (0.42 nmol/mg protein $^{-1}$ for IMP vs 4.3 for AMP, respectively). Although ATP was still the dominant adenine nucleotide (65% of the total adenine nucleotide pool), the total adenine nucleotide pool content was reduced to $\sim 65\%$ of prehypoxic values. In contrast to continuously oxygenated hearts, purine nucleosides and bases were detectable in hypoxic myocardium. However, since we used high flow hypoxia, most of these purine metabolites diffused into the extracellular space and into the venous effluent. The result of adenine nucleotide degradation coupled with the constant washout of nucleosides and bases during hypoxia resulted in a net decrease of 9.0 nmol/mg protein $^{-1}$ in the total purine pool. There were no significant changes in the contents of ATP, total adenine nucleotides, or total purines as a result of reoxygenation.

The effect of ischemia on adenine nucleotide, nucleoside, and purine base contents. We analyzed the tissue content of ATP and its hydrolysis products, including IMP, in hearts made ischemic for both 12 and 28 min. As observed for the

Table I. The Content of Adenine Nucleotides, Nucleosides, and Purine Bases in Rat Hearts

Groups	ATP	ADP	AMP	SAN	ado	ino	hypo	xan	S(NS + B)	Total
Control	31.1 (1.4)	4.4 (0.6)	1.6 (0.3)	37.0 (1.8)	tr	tr	tr	tr	tr	37.0 (1.8)
Hypoxia	15.8 (1.2)	4.6 (1.2)	4.3 (0.6)	24.2 (2.5)	2.0 (0.5)	0.2 (0.1)	1.6 (0.6)	tr	3.8 (1.0)	28.0 (2.5)
Hypoxia & reoxygenation	16.4 (1.7)	4.9 (1.2)	2.7 (1.4)	23.6 (3.1)	0.3 (0.2)	0.2 (0.1)	0.6 (0.7)	tr	1.0 (1.0)	24.6 (3.4)
12 min ischemia	6.4 (2.4)	5.5 (0.7)	8.4 (0.5)	20.3 (1.6)	1.7 (0.4)	1.1 (0.8)	13.4 (1.7)	1.6 (0.1)	17.8 (2.9)	38.1 (1.5)
12 min ischemia & reperfusion	17.2 (2.0)	2.8 (0.2)	1.2 (0.1)	21.5 (2.3)	1.1 (0.2)	0.2 (0.1)	0.1 (0.0)	0	1.3 (0.2)	22.5 (2.5)
28 min ischemia	1.9 (0.7)	2.5 (0.9)	13.7 (1.4)	18.1 (1.8)	2.2 (0.7)	2.1 (0.8)	14.2 (1.8)	2.0 (0.4)	20.5 (2.5)	38.6 (2.8)
28 min ischemia & reperfusion	12.5 (1.6)	2.8 (1.0)	2.2 (0.9)	17.5 (1.2)	0.6 (0.3)	tr	0.3 (0.4)	0	0.9 (0.5)	18.2 (1.6)

All values are mean \pm SD (in parentheses); for n in groups, see Methods. Metabolite contents are expressed as nanomoles per milligram protein $^{-1}$. SAN, sum of adenine nucleotides (ATP plus ADP plus AMP); ado, adenosine; ino, inosine; hypo, hypoxanthine; xan, xanthine; S(NS + B), sum of nucleosides (ado plus ino) and bases (hypo plus xan); tr, trace amount. Sums do not include values for IMP content.

comparison of ATP content measured by ^{31}P NMR and chemically in freeze-clamped tissue for hypoxic and reoxygenated hearts, ATP content measured by both methods for hearts made ischemic for 12 min did not differ significantly. Because the chemical technique is more sensitive than ^{31}P NMR spectroscopy, the decrease in ATP content in hearts made ischemic for 28 min could be quantitated chemically. In these hearts, ATP decreased by 94%, from 31.1 to 1.9 nmol/mg protein $^{-1}$.

The distribution of ATP hydrolysis products differs greatly in hypoxic and ischemic myocardium. After only 12 min of ischemia, the decrease in ATP, the increase in AMP, and the increase in diffusible purines are much greater than after 28 min of hypoxia. Unlike hypoxia where the dominant nucleotide remained ATP, the dominant nucleotide in ischemic myocardium was AMP. IMP content (1.1 nmol/mg protein $^{-1}$) was less than one-tenth of the AMP content (13.7 nmol/mg protein $^{-1}$). Unlike hypoxic myocardium, where the sum of purine nucleosides and bases was small compared to the total purine pool (13%), in ischemic myocardium the sum of purine nucleosides and bases was 50% of the total purine pool. In spite of these differences in the distribution of phosphorylated and nonphosphorylated purines during no-flow ischemia, the total purine pool was unchanged. The only significant effects of increasing time of ischemia from 12 to the 28 min were a greater decrease in ATP and a greater increase in AMP contents. Importantly, after 28 min of ischemia the major nucleotide present is AMP (76% of total adenine nucleotide pool).

Upon reflow, as expected, we observed the loss of the diffusible pool of nucleosides and bases. The sum of adenine nucleotides was preserved. In agreement with results from the ^{31}P NMR experiments, in hearts made ischemic for 28 min the ATP content measured chemically increased with reperfusion. Importantly, by comparing the changes in ATP, ADP, and AMP measured at the end of 28 min of ischemia and at the end of reflow, we found that the increase in ATP (10.6 nmol/mg protein $^{-1}$) equals the decrease in AMP (11.5 nmol/mg protein $^{-1}$). Even after 12 min of ischemia the decrease in AMP (7.2 nmol/mg protein $^{-1}$) accounts for 67% of the increase in ATP (10.8 nmol/mg protein $^{-1}$).

Evidence for accumulation of AMP in ischemic myocardium

The results presented above show that reperfusion is a bioassay of the ability of the ischemic heart to resynthesize ATP from AMP that had accumulated in cytosol. Our results show that the increase in AMP measured in acid extracts prepared from ischemic tissue is primarily cytosolic not bound to macromolecules. Finally, our results show that AMP accumulates to high concentrations in ischemia but not hypoxia.

These conclusions are supported by changes in signal intensity in the region of the ^{31}P NMR spectrum containing the resonance for AMP. The position of the AMP resonance is pH sensitive; the chemical shift is 6.0 ppm for pH near 7 and 4.7 ppm for pH near 6.2. As shown in Fig. 3, no identifiable AMP resonance was observed for control hearts, and only a small resonance at the appropriate chemical shift was observed for hypoxic hearts, but high signal intensity at the chemical shift appropriate for pH near 6.2 was present in the spectrum for the ischemic heart. Importantly, the intensity at the chemical shift for AMP rapidly decreased upon reperfusion. Since this region of the ^{31}P NMR spectrum may also contain resonances for glycolytic intermediates, which accumulate during ischemia and which also display pH-sensitive chemical shifts, we cannot

Table II. Cytosolic Concentrations of Unbound ATP, ADP, AMP, Pi, and pH_i in Control, Hypoxic, and Ischemic Rat Hearts

Group	ATP <i>mM</i>	ADP μM	AMP μM	Pi <i>mM</i>	pH _i
Control	10.8	39	0.2	5.0	7.10
4 min hypoxia	9.4	73	0.5	9.7	7.10
8 min hypoxia	8.7	148	2.6	14.8	7.06
12 min hypoxia	8.2	134	2.4	17.1	7.02
16 min hypoxia	7.7	147	3.0	17.0	7.01
4 min ischemia	9.5	147	2.5	19.7	6.74
8 min ischemia	5.8	284	14.0	21.5	6.38
12 min ischemia	3.4	767	143.0	26.5	6.24

All values are means from all hearts; for *n* in groups, see Methods.

assign all of this signal intensity to AMP. Therefore, these changes cannot be used quantitatively. However, ^{31}P NMR spectra of extracts prepared from these hearts (not shown) show a similar pattern, with the greatest intensity for the resonance identified as AMP in ischemic, much less in hypoxic, and least in control and reperfused hearts. Thus, consistent with the chemical assays, we conclude that AMP accumulates to millimolar amounts in ischemic myocardium and is rephosphorylated to ATP (AMP \rightarrow ADP \rightarrow ATP) during reperfusion.

The equilibrium expressions for creatine kinase and adenylate kinase can be used to estimate cytosolic concentrations of ADP and AMP, respectively. The creatine kinase equilibrium expression cannot, however, be used quantitatively for highly acidic pH's. The adenylate kinase equilibrium expression is also mildly pH dependent. Accordingly, estimates for cytosolic ADP and AMP were calculated in this way only for the first 16 min of hypoxia and the first 12 min of ischemia (Table II). Also shown are values for ATP, Pi, and pH_i obtained from the ^{31}P NMR experiments. Directionally consistent with results obtained from both ^{31}P NMR and chemical assay, cytosolic AMP increased \sim 15-fold during 16 min of hypoxia. Note that there is little change in the concentration of either ADP or AMP between 8 and 16 min of hypoxia. During ischemia, the increases in calculated ADP and AMP concentrations were even greater; by 12 min of ischemia there was a 20-fold increase in ADP and a 790-fold increase in AMP content. Thus, in spite of the uncertainty in equilibrium constants used for the creatine kinase and adenylate kinase reactions for the pH values observed in ischemia, ^{31}P NMR, chemical assay, and calculated values all show that the increase in AMP in ischemia is substantial and much greater than in hypoxia.

Discussion

In vivo regulation of 5'-NT

Recent studies of mammalian and avian myocardium have identified a cytosolic AMP-specific 5'-NT that catalyzes the unidirectional reaction of dephosphorylation of AMP to adenosine and Pi. This enzyme is distinct from both the cytosolic IMP-specific 5'-NT and ecto-AMP-specific 5'-NT (8–10). In vitro characterization studies have found that the cytosolic AMP-specific 5'-NT enzyme shows a 15-fold preference for AMP over IMP, requires Mg $^{2+}$, and has its optimum activity at

pH near 7 (8, 9). The K_m for AMP, 4.7 mM (9), is high relative to typical cytosolic AMP concentrations. However, little is known about the mechanisms regulating cytosolic 5'-NT activity in vivo in well-oxygenated, hypoxic, or ischemic conditions. By combining the tools of ^{31}P NMR spectroscopy and conventional chemical assay of ATP and its hydrolysis products, we have assessed the likely candidates for the activators and inhibitors of 5'-NT activity in hypoxic and ischemic hearts in vivo. Using this approach, we not only measured time-dependent changes in substrate and product but also defined changes in two important inhibitors of the 5'-NT reaction, P_i and H^+ . These data allow us to estimate the reaction velocity of 5'-NT in vivo and to assess the relative contributions of P_i and H^+ to enzyme regulation. Our results show that 5'-NT is activated during hypoxia and early during ischemia but is inhibited later during ischemia as pH_i decreased to approximately below 6.2.

Rat ventricular tissue contains both AMP- and IMP-specific 5'-NTs as well as AMP deaminase (8–11), all of which could contribute to adenine nucleotide degradation during hypoxia or ischemia. Although both IMP and AMP increase manyfold during hypoxia and ischemia, the AMP concentration was always an order of magnitude higher. Thus, it seems likely that neither IMP-specific 5'-NT nor AMP deaminase activities makes a major contribution to adenine nucleotide degradation in mammalian myocardium. Using another species, Swain et al. (16) also observed only a small accumulation of IMP during ischemia in dog heart. Similarly Geisbuhler et al. (21) found relatively low levels of IMP in rat hearts exposed to control (0.3 ± 0.5 nmol/mg protein $^{-1}$) and ischemic (1.1 nmol/mg protein $^{-1}$) conditions, with values similar to those reported here. Therefore, taken together, these results support the conclusion that dephosphorylation of AMP to adenosine is the major pathway converting adenine nucleotide to nucleoside in hypoxic and ischemic mammalian myocardium.

Velocity of 5'-NT in vivo

In *in vitro* experiments, the activity of 5'-NT is calculated from the rate of adenosine production from a constant supply of AMP. To estimate the activity of 5'-NT in vivo, substrate must be available and an estimate of the amount of product must be made. Since adenosine is rapidly degraded to inosine, hypoxanthine, and xanthine, the sum of purine nucleosides and nucleobases produced must be used to estimate the rate of product generated in vivo.

In control hearts, the concentration of the substrate, AMP, calculated from creatine kinase and adenylate kinase equilibrium expressions, is low, ~ 0.2 μM , but the relatively high concentrations of ADP and ATP serve as a large source of substrate. We were not able to detect either adenosine or its major catabolites, inosine and hypoxanthine, in either the myocardium or the coronary effluent (unconcentrated) of well-oxygenated hearts, showing that they are present in only low concentrations. Since the concentration of the total adenine pool is constant during normoxic perfusion, the rate of loss of purine from the myocardium should be equal to the rate of *de novo* synthesis of the adenine ring. Using values measured by Zimmer et al. (24) in isolated perfused rat hearts, we estimated this value to be 0.15×10^{-3} nmol/mg protein $^{-1}$ per min $^{-1}$.

Throughout hypoxia, the cytosolic concentration of ATP (measured) remained in the millimolar range, the ADP concentration (calculated) increased 3-fold from 39 to 147 μM ,

and the AMP concentration (calculated) increased ~ 15 -fold from 0.2 to 3 μM . If the reported K_m for AMP of 5'-NT of 4.7 mM applies *in vivo*, we would expect that the 15-fold increase in AMP concentration would increase the velocity of the 5'-NT reaction in hypoxia. Because flow is maintained during hypoxia the transsarcolemmal adenosine gradient remains high. Thus, we would expect that product removal would drive the reaction to completion in hypoxia but not during no flow ischemia.

During 28 min of hypoxia, ~ 12.8 nmol purine/mg protein $^{-1}$ (9.8 nmol/mg protein $^{-1}$ lost plus 3.8 nmol/mg protein $^{-1}$ remaining) were made from AMP, yielding an average reaction velocity of 0.46 nmol/mg protein $^{-1}$ per min $^{-1}$. Thus the estimated velocity of 5'-NT in hypoxic hearts is several orders of magnitude greater than for well-oxygenated hearts.

Unlike hypoxia, the estimation of the activity of 5'-NT during ischemia showed biphasic pattern. After 28 min of ischemia, the ATP concentration fell to < 1 mM, the ADP concentration increased to values close to the millimolar range, and the unbound AMP concentration (the maximum value calculated from the increase in AMP measured chemically) increased to ~ 4 mM. This AMP concentration, approximately equal to the K_m for the substrate for the 5'-NT, should drive the 5'-NT reaction at 50% its maximal rate. The average reaction rate calculated from the sum of nucleosides and nucleobases during the first 12 min of ischemia is 1.48 nmol/mg protein $^{-1}$ per min $^{-1}$. This value is three times higher than observed in hypoxia, is several orders of magnitude higher than for well-oxygenated heart, and agrees with other reports showing increased 5'-NT activity in ischemic leukocytes (25).

Two experimental observations described here, however, suggest that 5'-NT is no longer active after 12 min of ischemia. First, extending the duration of ischemia from 12 to 28 min did not alter the size of the adenine nucleotide pool. Instead, only the distribution of adenine nucleotides changed, characterized by a marked increase in AMP concentration. Second, increasing the duration of ischemia from 12 to 28 min did not significantly change the sum of nucleosides and bases present in this closed system. Thus, although the velocity of AMP-specific cytosolic 5'-NT increased during hypoxia and early ischemia, 5'-NT was not active during prolonged ischemia in spite of increased accumulation of substrate.

Inhibitors of 5'-NT in vivo

Results presented here show that inhibition of 5'-NT activity occurs by 12 min of ischemia in the isolated rat heart. The known inhibitors of 5'-NT are P_i and H^+ . Consistent with feedback inhibition by a product, accumulation of P_i inhibits 5'-NT activity. *In vitro* experiments have shown that 20-mM P_i inhibits 5'-NT activity by $\sim 30\%$ (8). We observed large increases in P_i during both hypoxia and ischemia, from 5 mM in well-oxygenated buffer-perfused hearts to 17 mM in hypoxic hearts and to ~ 40 mM in ischemic hearts. Partial inhibition of 5'-NT activity by high concentrations of P_i thus may explain the modest increase in AMP concentration during hypoxia and contribute to the large increase in AMP during ischemia.

Experiments with purified soluble 5'-NT from mammalian and avian hearts have shown that acidosis is the most potent inhibitor of the enzyme (8, 9). In the presence of ATP, ADP, and AMP, *in vitro* studies have shown a marked pH dependence: $\sim 75\%$ of maximal 5'-NT activity at pH 7.0, 50% at pH ~ 6.5 , and 25% at pH 6.2 (9). By comparing results for isch-

emia where both Pi and H⁺ increase with results for hypoxia where only Pi increases, we can separate the effects of inhibition by Pi from inhibition by H⁺. During the first 12 min of ischemia, the concentrations of the adenine nucleotides and Pi are not very different from those observed during 8 to 28 min of hypoxia. This observation makes it unlikely that changes in the concentrations of ATP, ADP, AMP, Mg²⁺ (which is released as MgATP is hydrolyzed), or Pi, alone or in combination, lead to significant inhibition of 5'-NT activity. The major quantitative difference between hypoxia and ischemia is the difference in pHi; in hypoxia pHi remained near 7 but fell to values close to 6.2 after 12 min of ischemia. This increase in H⁺ concentration in ischemia is sufficient to inhibit 5'-NT activity in vitro. Thus, results presented in this report provide evidence that the combination of intracellular acidosis and increased Pi is sufficient to inhibit 5'-NT during myocardial ischemia in vivo.

Role of 5'-NT inhibition on adenine nucleotide metabolism during recovery from hypoxia and ischemia

The ATP concentration during reoxygenation after hypoxia and during reperfusion after total ischemia is equal to the sum of any ATP remaining in the tissue plus ATP resynthesized from cytosolic ADP and AMP. Although the percent increase in AMP was large during hypoxia, the absolute amount was too small to contribute significantly to net resynthesis of ATP upon reoxygenation. Consequently, the amount of ATP in reoxygenated tissue was the same as the amount present at the end of hypoxia. In contrast, during reperfusion after ischemia ATP concentration increased rapidly. After 28 min of ischemia all of the AMP that had accumulated during ischemia was used for ATP resynthesis. The absolute amount of ATP synthesized by rephosphorylation of AMP was large, ~ 40% of the preischemic ATP content. Moreover, essentially all of the ATP was resynthesized during the first 4 min of reflow. This observation suggests that rephosphorylation of AMP is a much faster process than reactivation of 5'-NT activity.

Our results suggest that inhibition of a single enzyme, 5'-NT, is sufficient to account for the amount of net ATP resynthesis after prolonged ischemia. Sustained 5'-NT activity, converting AMP to adenosine during hypoxia, is also sufficient to account for the lack of net ATP resynthesis observed in reoxygenation after hypoxia.

Our observations provide new insight into several previous studies. In 1973 Bing et al. (26) investigated the influence of acidosis on contracture in papillary muscles isolated from hypoxic rat hearts. These authors observed rapid and complete mechanical recovery during reoxygenation when muscles were made hypoxic at acidic pH but not neutral pH. They attributed the protective role of acidosis to reduction of contractile force during hypoxic insult. Nayler et al. (27) observed that isolated rabbit hearts exposed to hypoxia with mild acidosis (pH ~ 6.8) had greater recovery of tension development during reoxygenation compared to hearts exposed to pH 7.4. Recently, Koop and Piper (28) demonstrated that acidosis (pH ~ 6.8) had energy-conserving effects on hypoxic myocytes. Rouslin and co-workers (29) have proposed a mechanism for the protective role of acidosis during ischemia. They suggest that inhibition of mitochondrial ATPase activity by H⁺ slows the rate of tissue ATP depletion in canine cardiac muscle. However, results obtained by Das and Harris (30) using rat myocytes showed that mitochondrial ATPase activity does not fall even in cells exposed to anoxia and pH ~ 5.0. None of these

studies addressed the role of acidosis on 5'-NT activity. Based on the observations made in this report, we suggest that one mechanism underlying these observations of improved mechanical performance and energy-sparing is inhibition of 5'-NT activity by H⁺ leading to increased ATP resynthesis from AMP.

He et al. (31) have addressed the role of cytosolic 5'-NT on adenosine production in isolated guinea pig hearts stressed by hypoperfusion with β -receptor stimulation and in rat hearts supplied with 2-deoxyglucose to reduce ATP content. They concluded that pHi is not an important regulator of 5'-NT in vivo; however, they did not successfully reproduce in vivo conditions necessary to inhibit the enzyme, namely profound acidosis.

Since the original report of Murry et al. (7) studying ischemia in the dog heart, work from several laboratories has demonstrated that exposure of the myocardium to several brief periods of ischemia leads to increased tolerance for subsequent sustained coronary artery occlusion. In spite of much research, the mechanism(s) underlying the phenomenon of preconditioning are not fully defined. A number of investigators have found that ATP content falls only during the first occlusion (32-34). Since a significant fall in pHi can occur within the first few minutes of occlusion (13), we speculate that acidosis attenuates adenine nucleotide degradation and adenosine production in subsequent periods of ischemia (long or short) because 5'-NT activity is inhibited. Therefore, inhibition of 5'-NT may contribute to the preconditioning phenomenon.

Although our observations were made in the isolated rat heart, our findings suggest a mechanism by which severe acidosis attenuates adenine nucleotide depletion in vivo in other ischemic organs as well. Studying isolated kidneys, Stromski et al. (35) observed that the magnitude of ATP recovery increased with the size of residual adenine nucleotide pool at the end of the ischemic period and that this increased ATP correlated with improved functional recovery. Our results suggest a mechanism for these observations. By inhibiting 5'-NT activity, not only is AMP increased (leading to resynthesis of ATP during reperfusion) but also production of adenosine is decreased. Since adenosine release during ischemic injury causes vasoconstriction in kidneys, inhibition of 5'-NT activity by acidosis may have a dual protective role in ischemic injury in the kidney.

In summary, our results suggest the mechanism whereby previously ischemic tissue resynthesizes ATP during reperfusion: inhibition of 5'-NT activity in ischemic tissue by intracellular H⁺ attenuates depletion of adenine nucleotide pool. In this way the capacity of reperfused tissue to resynthesize ATP is increased. These observations may have several clinical implications.

Clinical implications

Cardioplegia. It is currently accepted that to obtain maximum recovery cardioplegic solutions should be alkaline (pH ~ 7.8). However, Bernard et al. (36) observed higher ATP levels and better functional recovery of ischemic rat myocardium preserved with slightly acidic cardioplegic solution (pH ~ 7.0) as compared with alkaline cardioplegic solution (pH 7.8). Iannetoni et al. (37) have recently confirmed these observations by demonstrating that postischemic recovery of ventricular function was significantly improved in hearts arrested with acidic cardioplegic solution (pHi ~ 6.3) as compared with hearts arrested with pHi ~ 7.8; intermediate recovery was ob-

served for buffer at $\text{pH}_i \sim 7.0$. We suggest that the basis for these results, at least in part, is preservation of the adenine nucleotide pool during ischemia by H^+ inhibition of 5'-NT activity. Taken together, these results suggest that defining the optimal pH for cardioplegic solutions merits further investigation.

Ischemia and infarction. Occlusion of the coronary artery and prolonged ischemia result in spatial and temporal inhomogeneities in flow, mechanical function, and viability. Measurements of regional myocardial blood flow demonstrate time-dependent heterogeneity of local flow in the area affected by the ischemic insult, ranging from no flow to above normal flows (38–41). Our results studying hypoxia and ischemia in experimental models provide a description of some of the biochemical events that may occur in these ischemic regions with varying flows. Partial reductions in coronary flow result in ATP degradation. Although maintaining flow ensures delivery of metabolic substrates it is not sufficient to maintain normal ATP levels. Maintaining flow also ensures washout of the end products of metabolism, including H^+ , but it also results in continual washout of purine nucleosides and bases. Our results studying high flow hypoxia suggest that production and loss of purines from these regions are primarily due to increased 5'-NT activity. Because of the sustained activity of 5'-NT, AMP does not accumulate. Consequently, these regions of the myocardium are at risk in terms of their ability to recover normal ATP levels when normal flow and metabolism are restored. In contrast, in regions of no or very low flow, accumulation of H^+ is sufficient to inhibit 5'-NT activity, leading to AMP accumulation. If these areas are reperfused before the cells are irreversibly injured, this AMP pool serves as a substrate for net ATP resynthesis. In our experimental model of ischemia with reperfusion, we did not observe complete recovery of ATP, however.

Thus, the reperfused regions and the surrounding area at risk (stunned myocardium) both contain ATP, but at reduced levels. Until ATP can be resupplied via de novo synthesis of the purine ring (a slow process requiring days), both of these regions require careful clinical management to minimize ATP utilization. The delay in return to normal ATP levels may also delay return of cardiac function. It is relevant that increasing flow during ischemia by pharmacological interventions has been shown to improve function only slightly (42) or not at all (43).

Thus, our results suggest that there can be heterogeneity in 5'-NT activity in ischemic regions with varying flows ranging from activation to inhibition. The consequences of these regional differences are variations in the amount of adenosine production during ischemia and in the capacity for net ATP resynthesis upon reperfusion.

Finally, our data suggest that the perception of the toxicity of intracellular acidosis during ischemia should be modified. While prolonged acidosis has many harmful consequences, our results suggest that acidosis during no-flow ischemia protects against organ ATP depletion and thus may ameliorate reperfusion injury.

Acknowledgments

We thank Ilana Reis for her excellent technical assistance, Dr. Rolf Büniger for helpful discussions, and Sandra Browne for assistance in manuscript preparation.

This research was supported by National Institutes of Health grant HL-43170.

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