Effect of Ischemia on Adenine Nucleotides in Cardiac and Skeletal Muscle

By Shoichi Imai, M.D., Ph.D., Arthur L. Riley, and Robert M. Berne, M.D.

In the isolated cat heart and in the working heart of the open-chest dog, severe hypoxia resulted in the appearance of inosine and hypoxanthine in the perfusate and coronary sinus blood, respectively.1-3 On the basis of these observations and because adenosine is rapidly converted to inosine and hypoxanthine by blood and by perfusates,1 the hypothesis was proposed that with myocardial hypoxia, adenosine is formed from the breakdown of the cardiac adenine nucleotides, diffuses out of the myocardial cells, and is responsible for the accompanying reduction of coronary vascular resistance.2,3 It was also postulated that adenosine is the physiological agent involved in the intrinsic regulation of coronary blood flow. However, it has not been possible to demonstrate adenosine in the cardiac effluent, possibly because of rapid enzymatic degradation of adenosine or possibly because adenosine is not formed in the myocardium and the recovered inosine and hypoxanthine arise from inosinic acid which appears as the result of deamination of adenyllic acid within the myocardial cells. The present studies were undertaken to determine whether adenosine is one of the intermediates in the degradation of the cardiac adenine nucleotides associated with hypoxia and, if so, whether it readily leaves the cardiac cells. Parallel studies were done on skeletal muscle since blood flow regulation in this tissue also appears to be linked with metabolic processes.

Methods

CONTROL MUSCLE SAMPLES

The anterior tibialis muscles of both legs of rabbits (2.5 to 3 kg, anesthetized with urethane 1.5 g/kg iv) were carefully exposed and the central portions of the muscles instantly frozen in situ by means of Wollenberger tongs4 precooled in liquid nitrogen. The maximal thickness of the frozen muscle samples was 1.5 mm. These "wafers" of frozen skeletal muscle were kept in liquid nitrogen until analytical procedures were started. Under artificial respiration, the rabbit's chest was opened in the midline to expose the heart. After a delay of about 10 minutes to ensure the establishment of a new steady state of the cardiovascular system, the ventricles of the heart were cut free with a pair of scissors and instantly frozen by means of the Wollenberger tongs. The time interval between the removal and freezing of the ventricles was less than two seconds. As in the case of the skeletal muscle samples, the frozen ventricles were also stored in liquid nitrogen.

HYPOXIC AND ISCHEMIC MUSCLE SAMPLES

Two types of experiments were performed with heart muscle. In one series, severe myocardial hypoxia was produced by stopping artificial respiration in the open-chest animal. Following various periods of respiratory arrest, the ventricles were cut free and frozen in the same manner as described for the control experiments. In the second series of experiments on rabbit myocardium, the ventricles were rapidly removed during artificial respiration, cut into three sections, placed in a moist chamber, and incubated at 37.0°C as described by Gerlach et al.5,6 The sections of myocardium were sequentially removed and frozen after varying periods of incubation. The skeletal muscle samples were quickly excised, cut into four sections, and treated in the same manner as the incubated sections of heart muscle.
EXTRACTION PROCEDURE AND CHEMICAL ANALYSES

The frozen muscle "wafers" were finely powdered in a hollow stainless steel cylinder fitted with a stainless steel piston, both precooled in liquid nitrogen. The pulverized muscles were then transferred to a tared Potter-Elvehjem homogenizer tube, which had been immersed in powdered dry ice. Based on an estimate of the tissue weight, approximately one volume of 0.6 N perchloric acid (PCA) had been added and frozen around the bottom wall of the tube prior to the initial weighing. The tube was reweighed after addition of the muscle powder and the weight of the muscle was determined by difference. Just before homogenization, the tube was transferred to an ice bath and cold 0.6 N PCA was added until the total volume of PCA equaled twice the weight of the muscle sample. Usually a part of the PCA, primarily the first portion layered upon the powder, became frozen under these conditions. The mixture was homogenized for 10 minutes with a Teflon pestle, during which time the frozen extracting agent at the bottom of the tube gradually melted and penetrated the thawing muscle powder. The precipitated protein was centrifuged with an International refrigerated centrifuge for 10 minutes at 2000 rev/min at 0°C, in the original homogenizer tube. The resulting clear supernatant fluid was decanted into a beaker placed in an ice bath and the residue was homogenized again with two volumes of cold 0.2 N PCA for three minutes and recentrifuged. The combined supernatant fractions were carefully neutralized at pH 7 by the addition of KOH. An aliquot of the neutralized extract was used for direct enzymatic determination of ATP, ADP, AMP, adenosine, inosine, and hypoxanthine. The remainder was frozen and stored at −20°C until subjected to ion-exchange chromatography.

The adenine nucleotides were measured fluorometrically by the firefly method in an Aminco-Bowman spectrophotofluorometer. ATP was measured directly and ADP and AMP after enzymatic conversion to ATP by the following reactions:

For ADP:

\[
\text{ADP} + \text{phosphoenolpyruvate (PEP)} \xrightarrow{\text{kinase}} \text{pyruvate}
\]

For AMP:

\[
\text{AMP} + \text{ATP} \xrightarrow{\text{myokinase}} 2 \text{ADP}
\]

These reactions were accomplished at room temperature for 30 minutes in a mixture of the following composition: glycylglycine buffer (pH 7.5) 25 mM, MgSO₄ 7.5 mM, KCl 75 mM and PEP* 4 mM. The pyruvate kinase and myokinase were purchased as ammonium sulphate suspensions and diluted with 10% aqueous solution of bovine plasma albumin just before use. AMP was also determined on the neutralized extract from the change in optical density at 265 µM upon deamination to inosine monophosphate (IMP).* Adenosine, inosine, and hypoxanthine were quantitated enzymatically by the methods of Kalckar. Adenosine deaminase was prepared from calf alkaline phosphatase, and nucleoside phosphorylase, from rat liver. Xanthine oxidase was purchased as an ammonium sulfate suspension and was used as an ammonium sulfate suspension just prior to use.

For the determination of IMP, the extracts of two or three samples were combined and placed on a Dowex 1 (100 × 200 mesh) column (1.0 × 12.0 cm) in the bi-carbonate form. The nucleotides and their derivatives were separated by gradient elution with potassium bicarbonate. IMP was identified by comparisons of the chromatographic pattern and spectral ratio (250/260 nm) of unknown samples with pure samples of IMP. Quantitation of IMP was done by ultraviolet absorption at 248 nm using a molar extinction coefficient of 12.3 × 10³ and by enzymatic assay of the hypoxanthine formed after hydrolysis with 5 N H₂SO₄ for four hours at 100°C.

Inorganic phosphate was selectively precipitated with a suspension of Ca (OH)₂ in CaCl₂ and determined by the method of Fiske and Subbarow. Total phosphate (inorganic phosphate plus creatine phosphate) was determined on a second aliquot of the neutralized muscle extract by incubation with the acid-molybdate reagent for 30 minutes at room temperature. The creatine phosphate was estimated as the difference between total and inorganic phosphates. According to Wahler and Wollenberger, there are no acid-molybdate-labile phosphates in heart muscle other than creatine phosphate.

Results

In table 1 are listed the amounts of the

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* Sigma Chemical Company.
† Nutritional Biochemicals Corporation.
ISCHEMIA AND MUSCLE NUCLEOTIDES

### TABLE 1

Adenine Nucleotides and Related Compounds in Heart and Skeletal Muscle (nmole/g wet wt)*

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Heart muscle (n = 7)</th>
<th>Skeletal muscle (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP</td>
<td>0.17 ± 0.07</td>
<td>0.29 ± 0.08</td>
</tr>
<tr>
<td>ATP</td>
<td>4.04 ± 0.15</td>
<td>6.30 ± 0.25†</td>
</tr>
<tr>
<td>ADP</td>
<td>0.67 ± 0.14</td>
<td>0.76 ± 0.07</td>
</tr>
<tr>
<td>Adenosine</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Inosine</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>IMP</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>Creatine phosphate</td>
<td>7.11 ± 0.27</td>
<td>23.14 ± 1.41†</td>
</tr>
<tr>
<td>Inorganic phosphate</td>
<td>4.05 ± 0.41</td>
<td>4.51 ± 0.80</td>
</tr>
<tr>
<td>ATP/AMP ratio</td>
<td>7.11 ± 0.27</td>
<td>23.8</td>
</tr>
</tbody>
</table>

* Mean ± standard error.
† P < 0.05.
‡ P < 0.01.

n.d.: not detected.

Detected in normal cardiac or skeletal muscle and IMP is present only in skeletal muscle and in small amounts. The high levels of creatine phosphate, the low concentration of inorganic phosphate, and the high ATP/AMP ratios (considered by some investigators to be a more sensitive indicator of the degree of ATP degradation than the more commonly used ATP/ADP ratio) seem to indicate the adequacy of the method employed in removing and freezing the tissues.

In experiments in which cardiac excision was done at different times after interruption of the respirator, myocardial ATP and creatine phosphate were slightly reduced at one minute and markedly reduced at two and three minutes (table 2). Associated with these changes was an accumulation of ADP and AMP, an increase in inorganic phosphate and a decrease in the ATP/AMP ratio from twenty-four to six within three minutes. Traces of adenosine, inosine, hypoxanthine, and IMP were found in the myocardium of hypoxic hearts with intact coronary circulation.

Since coronary blood flow continues during the period of hypoxemia and is initially greatly increased by virtue of the reduced coro-

### TABLE 2

Adenine Nucleotides and Related Compounds in Hypoxic Perfused Heart Muscle (nmole/g wet wt)*

<table>
<thead>
<tr>
<th>Compounds</th>
<th>1 min (n = 5)</th>
<th>2 min (n = 6)</th>
<th>3 min (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>3.69 ± 0.28</td>
<td>2.51 ± 0.16</td>
<td>2.37 ± 0.19</td>
</tr>
<tr>
<td>ADP</td>
<td>0.88 ± 0.18</td>
<td>1.36 ± 0.10</td>
<td>1.29 ± 0.24</td>
</tr>
<tr>
<td>AMP</td>
<td>0.29 ± 0.04</td>
<td>0.28 ± 0.04</td>
<td>0.37 ± 0.07</td>
</tr>
<tr>
<td>Adenosine</td>
<td>n.d.</td>
<td>tr.</td>
<td>tr.</td>
</tr>
<tr>
<td>Inosine</td>
<td>n.d.</td>
<td>tr.</td>
<td>tr.</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>tr.</td>
<td>tr.</td>
<td>tr.</td>
</tr>
<tr>
<td>IMP</td>
<td>n.d.</td>
<td>tr.</td>
<td>tr.</td>
</tr>
<tr>
<td>Creatine phosphate</td>
<td>6.62 ± 1.11</td>
<td>1.67 ± 0.91</td>
<td>0.70 ± 0.32</td>
</tr>
<tr>
<td>Inorganic phosphate</td>
<td>3.88 ± 0.50</td>
<td>8.48 ± 0.58</td>
<td>9.00 ± 0.75</td>
</tr>
<tr>
<td>ATP/AMP ratio</td>
<td>12.7</td>
<td>9.0</td>
<td>6.4</td>
</tr>
</tbody>
</table>

* Mean ± standard error.
† P < 0.05.
‡ P < 0.01.
tr. = trace.
n.d.: not detected.

Adenine nucleotides and their derivatives in normal rabbit heart (ventricles) and skeletal muscle. Skeletal muscle contains much larger quantities of creatine phosphate and 56% more ATP than heart muscle, whereas the amounts of ADP and AMP are approximately equal in these two types of muscle. Adenosine,* inosine, and hypoxanthine could not be detected in normal cardiac or skeletal muscle and IMP is present only in skeletal muscle and in small amounts. The high levels of creatine phosphate, the low concentration of inorganic phosphate, and the high ATP/AMP ratios (considered by some investigators to be a more sensitive indicator of the degree of ATP degradation than the more commonly used ATP/ADP ratio) seem to indicate the adequacy of the method employed in removing and freezing the tissues.

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*The preliminary report that adenosine is present in normal myocardium (Physiologist 6: 138, 1963) was incorrect because of a previously unrecognized technical error in the method.
nary resistance and increase in arterial pressure induced by asphyxia, it was conceivable that products of adenine nucleotide degradation were being washed out of the myocardium. Therefore, experiments were performed on anoxic unperfused cardiac muscle. It was at this time in our studies that the reports of Gerlach et al. on the effect of anoxia on cardiac nucleotides appeared and we adopted their technique. In these experiments, the changes in the adenine nucleotides were essentially the same as those in which the coronary circulation was intact (figs. 1, 2, and 3). However, there were differences in the myocardial concentrations of adenosine, inosine, hypoxanthine, and IMP with cardiac ischemia (fig. 3). Adenosine, absent in control samples and after one minute of ischemia, reached a peak value within 2.5 minutes and then declined during the 20-minute period of observation. Associated with the changes in myocardial adenosine were progressive increases in the tissue concentration of inosine and hypoxanthine. Initially, IMP rose with adenosine but remained elevated at a time when the adenosine concentration had decreased.

In skeletal muscle subjected to the same ischemic conditions, the ATP concentration was fairly well maintained, presumably at the expense of creatine phosphate which was decreased to almost one-half the normal levels after 30 minutes of ischemia (figs. 4

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**FIGURE 1**

Changes in the amounts of creatine phosphate (CrP) and inorganic phosphate (Pi) in ischemic heart muscle. Each point represents the average of six experiments. The bars represent standard errors of the means.

**FIGURE 2**

Adenine nucleotides in ischemic heart muscle.

**FIGURE 3**

Effect of ischemia on adenosine (Ada), inosine (In), hypoxanthine (Hx), and inosinic acid (IMP) concentrations in heart muscle.
and 5), ADP and AMP showed an early decrease, followed in the case of ADP by a smaller increase. In contrast to cardiac muscle, skeletal muscle was found to contain IMP and the concentration of this nucleotide increased with ischemia (fig. 6). Associated with the increase in IMP in the ischemic muscle was a progressive rise in the concentration of inosine and hypoxanthine. Adenosine was not detected in adequately oxygenated or anoxic skeletal muscle.

**Discussion**

The results of the present study on the effect of anoxia on the myocardial adenine nucleotides and their derivatives in the rabbit, essentially confirm the work of Gerlach et al.5, 6 in the rat. In their experiments the increase of adenosine was greater, lasted longer, and was associated with a slower increase of IMP. Whether this is a species difference or whether the slight discrepancies are due to differences in methodology cannot be ascertained. In the present study, specific enzymatic methods were employed to identify and quantitate the adenine compounds, whereas paper chromatography and ultraviolet absorption were essentially the means used by Gerlach et al.5, 6.

The appearance of adenosine in myocardium deprived of its blood supply is in harmony with the idea that adenosine may be the substance responsible for reactive hyper-

Inorganic phosphate

Creatine phosphate

![Figure 4](image_url)

**FIGURE 4**

Creatine phosphate and inorganic phosphate in ischemic skeletal muscle. Each point represents the average of six experiments. The bars represent standard errors of the means.

Adenine nucleotides in ischemic skeletal muscle.

![Figure 5](image_url)

**FIGURE 5**

Adenine nucleotides in ischemic skeletal muscle.
Effects of ischemia on inosine (In), hypoxanthine (Hx), and inosinic acid (IMP) concentrations in skeletal muscle. Adenosine was not detected.

The effects of ischemia on adenine nucleotides of skeletal and cardiac muscle were similar in many respects. However the following differences were observed:

1) A striking difference was the absence of adenosine in anoxic skeletal muscle. This finding indicates that it is unlikely that adenosine plays a role in the control of skeletal muscle blood flow analogous to that hypothesized for the coronary circulation.

2) A greater rate and magnitude of ATP reduction occurred in cardiac as compared to skeletal muscle under identical experimental conditions. This difference probably can be in large part accounted for by the greater store of creatine phosphate in skeletal muscle and in small measure to the persistence of cardiac contractions for variable periods of time after excision of the heart.

3) The concentrations of inosine and hypoxanthine in ischemic cardiac muscle were ten times greater than those in ischemic skeletal muscle; observations which are attributable to the formation of adenosine from ATP in myocardium and its rapid deamination by adenosine deaminase (present in large concentrations in ventricular muscle). The high degree of activity of the adenosine deaminase is also evident from the decrease in the myocardial adenosine levels after the first 2.5 minutes of ischemia.

4) A sharp decrease in skeletal muscle ADP and a small increase in myocardial ADP were produced by ischemia. The reasons for these changes are not immediately apparent. The reduction in skeletal muscle ADP may be due to direct irreversible deamination of ADP to IMP. This reaction could also account for a similar decrease in skeletal muscle AMP, since less ADP, formed from hydrolysis of ATP, would be available for conversion to AMP. Unfortunately, no attempt was made to determine the muscle IMP concentrations in the present study. Failure to observe a decrease in myocardial ADP with ischemia may be due to the fact that direct deamination of ADP to IMP does not occur in rabbit heart muscle.

5) IMP is present in skeletal muscle and absent in heart muscle. These findings may be due to considerably higher concentration of adenylic acid deaminase in skeletal muscle. Accumulation of IMP in skeletal and cardiac muscle under ischemic conditions, is possibly on the basis of insufficient available energy, since the first step in reamination of IMP to AMP is an energy-requiring reaction involving condensation of IMP with aspartate to yield

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adenylosuccinate. However, there is some doubt about the applicability of this explanation to heart muscle since adenylosuccinate synthetase activity has been reported to be absent in rabbit myocardium. The appearance of IMP in ischemic myocardium and its virtual absence in anoxic hearts with a functioning coronary circulation, suggest that the anoxic myocardial cell membrane may be permeable to IMP. It is also conceivable that the skeletal muscle sarcolemma is permeable to AMP, as suggested by Conway and Cooke, and that AMP may play a role in the metabolic regulation of skeletal muscle blood flow.

Summary

Rabbit skeletal muscle contains 56% more ATP and 225% more creatine phosphate than rabbit cardiac muscle. With ischemia the percent reduction in ATP and creatine phosphate is less in skeletal than cardiac muscle and ADP and AMP decrease in skeletal and increase in cardiac muscle. IMP is present in skeletal muscle and increases with ischemia, whereas it is absent in cardiac muscle but appears with ischemia. Adenosine, inosine, and hypoxanthine are absent in skeletal and cardiac muscle. Ischemia results in the appearance of adenosine, inosine, and hypoxanthine in cardiac muscle but only the latter two compounds appear in ischemic skeletal muscle. Although complete ischemia is required to demonstrate the presence of adenosine in cardiac muscle, the appearance of this nucleoside is nevertheless consistent with the hypothesis that under physiological conditions quantities of adenosine, undetectable by present methods, may play a role in the regulation of coronary blood flow.

Acknowledgment

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