Changes in Content of Purine Nucleoside in Canine Myocardium during Coronary Occlusion

By R. A. Olsson, M.D.

ABSTRACT

The use of a sensitive double-beam spectrophotometer and modifications in enzymatic assay techniques permitted the measurement of adenine nucleosides in extracts of canine left ventricle. In hearts not subjected to left coronary occlusion, adenosine content averaged 0.32 ± 0.04 (SE) nmole/g, increasing to an average of 1.82 ± 0.21 (SE) nmole/g after 15 seconds of coronary occlusion. The myocardial content of inosine plus hypoxanthine was 1.07 ± 0.21 (SE) nmole/g without coronary occlusion, rising to 1.98 ± 0.24 (SE) nmole/g after 15 seconds of coronary occlusion. Unlike the myocardial content of adenosine and inosine, the myocardial content of hypoxanthine did not appear to change during coronary occlusion.

ADDITIONAL KEY WORDS

adenosine inosine hypoxanthine

Coronary blood flow rate is extremely sensitive to myocardial oxygen demand, but the mechanism by which the metabolic state of the heart effects changes in coronary vascular tone is incompletely understood. According to one hypothesis (1), increases in myocardial oxygen demand lead to the degradation of adenine nucleotides to adenosine, which in turn causes coronary vasodilation. Consonant with this hypothesis, adenosine and its breakdown products, inosine and hypoxanthine, have been shown to accumulate in anoxic heart muscle (2, 3). These compounds have been recovered from the coronary sinus effluent of isolated hearts perfused with oxygen-poor solutions (4), and the rate of coronary perfusion in similar experiments appears to be a function of the amount of nucleoside recovered (5). Adenosine has been recovered from the coronary sinus effluent during myocardial reactive hyperemia (6), and recently it has been recovered from solutions bathing the epicardial surface of the normally oxygenated heart (7), which suggests that ischemia need not be the sole determinant of its appearance in the myocardium.

Until recently it has not been possible to estimate the minute amounts of adenine nucleoside present in the normally oxygenated heart except by indirect methods (cf. 6 and 7). However, spectrophotometers are now available (8) which are sensitive enough to permit measurements of these compounds directly in heart muscle extracts. This report describes the analytical techniques employed to make these estimates, and the changes in nucleoside content which occur in the canine left ventricle during intervals of left coronary occlusion of up to 15 seconds.

Materials and Methods1

Beagle dogs of either sex weighing 10 to 18 kg were anesthetized with pentobarbital, approximately 30 mg/kg iv. Artificial respiration was provided by a Harvard respirator pump through a cuffed endotracheal tube. The chest was opened

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1In conducting the research described in this report, the investigator adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences-National Research Council.

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in the fourth left interspace, and in most experiments, the fifth rib was removed. The left coronary artery was dissected and, in coronary occlusion experiments, a ligature was passed beneath the artery at its origin. Biopsy of the myocardium was done either without coronary artery ligation or at 5, 10 or 15 seconds after the ligature was tied. Biopsy was performed with specially modified Sauerbruch rib rongeurs which had been cooled in liquid nitrogen. The quick-frozen tissue thus obtained was pulverized in a stainless steel percussion mortar previously cooled in liquid nitrogen, and the tissue powder was transferred to a tared homogenizer tube containing 2.00 ml of shell-frozen 0.6M perchloric acid. In some experiments, the tissue sample was divided between two homogenizer tubes to assess the possible influence of sample handling variation on the results. The contents of the tubes were homogenized with cooling in a dry ice, acetone bath, and after rewarming, the tube was weighed to determine the weight of tissue added. The acid extract was separated by centrifugation and an aliquot of the supernatant was neutralized to pH 7.2 to 7.4 with 5M potassium carbonate. After cooling in an ice bath for 10 minutes, the potassium perchlorate precipitate was separated by a brief centrifugation, and two aliquots of the supernatant were pipetted into 1 cm silica cuvettes. If the tissue sample had been divided prior to homogenization, the neutralized extracts were not analyzed in duplicate. The volume of the assay mixture was brought to 3.00 ml by the addition of 0.1M Tris HC1, pH 7.4 containing 3 mM potassium monohydrogen phosphate and 3 mM EDTA. Hypoxanthine, inosine, and adenosine were assayed spectrophotometrically by the sequential addition of approximately 0.04 units of xanthine oxidase, 0.6 units of nucleoside phosphorylase and 10 units adenosine deaminase, respectively.

The changes in absorbance consequent to the addition of each enzyme were recorded with an Aminco-Chance dual wavelength split-beam recording spectrophotometer set to record the difference in absorbance between 255 nm, an absorption maximum for uric acid, and 317 nm, which was found in preliminary experiments to be an isobestic point for these reactants. All nucleoside assays were completed within 4 hours after the tissue sample was obtained.

The content of each nucleoside per gram of left ventricle (wet weight) was calculated by the following formula:

\[
\text{Nucleoside content} = \frac{A \times V_1}{E \times (V_2 + V_4) \times (2.00 + 0.8 \times \text{T.W.})} \times \frac{(V_1 + V_2) \times (2.00 + 0.8 \times \text{T.W.})}{\text{T.W.} \times (V_2 + V_4)},
\]

where: A = absorbancy change (cm⁻¹),
E (uric acid) = molar absorbancy of uric acid, 1.22 \times 10⁴ cm²mole⁻¹,
T.W. = weight of frozen tissue taken for analysis (g),
\(V_1\) = volume of reaction mixture in cuvette (liters),
\(V_2\) = volume of acid extract neutralized (ml),
\(V_3\) = volume of neutralized extract in reaction mixture (ml),
\(V_4\) = volume of potassium carbonate used for neutralization (ml),
0.8 = water content of cardiac muscle (ml/g, assumed).

In addition to the duplicate assays, the accuracy of the analytic methods was checked by recovery experiments of two types. Mixtures of known amounts of nucleoside were added to tissue extracts, and the extracts were then assayed in the usual manner. A typical experiment is described in Table 1. Recovery experiments were also performed by adding known amounts of nucleoside to assay cuvettes after the completion of an assay. This also served as a convenient check on the activity of the enzymes used.

Data on myocardial nucleoside content were included in the results if the following criteria were met: postmortem examination showed that biopsy was confined to the left ventricle; ligature was proximal to all branches of the left coronary artery; the jaws of the biopsy rongeur were completely closed at completion of tissue sampling, and finally, that the difference between duplicate assays was 0.3 nmole/g or less. However, the last requirement was not adhered to in compiling the data on reproducibility of the method; all assays, including some from unrelated experiments, were included in this evaluation.

**Results**

Heart rates ranged between 180 and 220 beats/min, and arterial blood pressure ranged between 110 and 160 mm Hg, declining less...
Results of duplicate assays of myocardial nucleosides of single homogenates. See text for details.

Data on duplicate assays of single neutral-

than 15 mm Hg during the intervals of coronary occlusion studied. The site of biopsy was relatively constant, lying midway between the base and apex of the free wall of the left ventricle, just lateral to the obtuse border. It was difficult to determine the depth of biopsy, but it was estimated that this varied from an occasional sample of just the outer half of the ventricular wall to a majority of samples containing tissue from both the outer and inner halves of the ventricular wall.

Data on duplicate assays of single neutral-
TABLE 1

Recovery of Purine Nucleosides from Extracts of Dog Blood

<table>
<thead>
<tr>
<th>Approx. cons of nucleosides (nmole/ml)</th>
<th>Hypoxanthine</th>
<th>Inosine</th>
<th>Adenosine</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>0.09 ± 0.01</td>
<td>0.06 ± 0.02</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td>0.1</td>
<td>0.17 ± 0.02</td>
<td>0.15 ± 0.02</td>
<td>0.13 ± 0.03</td>
</tr>
<tr>
<td>0.2</td>
<td>0.25 ± 0.11</td>
<td>0.26 ± 0.06</td>
<td>0.22 ± 0.02</td>
</tr>
<tr>
<td>0.5</td>
<td>0.49 ± 0.02</td>
<td>0.52 ± 0.07</td>
<td>0.50 ± 0.03</td>
</tr>
<tr>
<td>1.0</td>
<td>0.89 ± 0.05</td>
<td>1.01 ± 0.03</td>
<td>0.97 ± 0.13</td>
</tr>
</tbody>
</table>

Solutions of each nucleoside were made up and standardized spectrophotometrically, using the following millimolar extinction coefficients at the wavelength indicated: hypoxanthine, 10.6 at 250 nm; inosine, 12.2 at 248.5 nm; adenosine, 15.4 at 259 nm. These solutions, nominally 0.1 mM for each nucleoside, were found to be: hypoxanthine, 0.0804 mM; inosine, 0.0948 mM; adenosine, 0.0032 mM. From 10 to 100 µl of each was added to 10.0 ml aliquots of a perchloric acid extract of dog blood to give concentrations in the range listed in column 1. The extracts were then neutralized and assayed in quadruplicate. Values found in extracts to which no nucleoside had been added represent the endogenous nucleoside of blood.

The results of a typical recovery experiment are shown in Table 1. In this experiment, the amount of nucleoside found was in excellent agreement with the amount calculated. Only three of the 60 assays in this experiment differed by more than 0.1 nmole/ml, and these three results were grossly abnormal. The average recovery of 0.1 to 1.0 nmole of adenosine to assay cuvettes in 23 experiments was 1.07 ± 3 (SE) %.

Data on left ventricular nucleoside content are presented in Table 2. The adenosine content in experiments without coronary occlusion (control) was 0.32 nmole/g, rising to an average value of 1.82 nmole/g after 15 seconds of coronary occlusion. In the experiments on hearts not subjected to coronary occlusion and in some of those with 5 seconds of coronary occlusion, inosine and hypoxanthine were assayed together. The sum of

TABLE 2

Myocardial Adenine Nucleoside Concentration of the Left Ventricle during Coronary Occlusion (nmole/g)

<table>
<thead>
<tr>
<th>Occlusion time</th>
<th>Adenosine</th>
<th><em>P</em></th>
<th>Inosine</th>
<th>Hypoxanthine</th>
<th>Inosine + hypoxanthine</th>
<th><em>P</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (6)</td>
<td>0.32 ± 0.04</td>
<td></td>
<td></td>
<td></td>
<td>1.07 ± 0.21</td>
<td></td>
</tr>
<tr>
<td>5 seconds</td>
<td>0.72 ± 0.08</td>
<td>&lt;0.001</td>
<td>0.71 (3)</td>
<td>0.66 (3)</td>
<td>1.29 ± 0.08</td>
<td>NS</td>
</tr>
<tr>
<td>10 seconds</td>
<td>1.13 ± 0.09</td>
<td>&lt;0.01</td>
<td>1.13 ± 0.18 (5)</td>
<td>0.63 ± 0.16 (8)</td>
<td>1.56 ± 0.28</td>
<td>NS</td>
</tr>
<tr>
<td>15 seconds</td>
<td>1.52 ± 0.21</td>
<td>&lt;0.01</td>
<td>1.44 ± 0.13 (7)</td>
<td>0.72 ± 0.18 (7)</td>
<td>1.98 ± 0.34</td>
<td>NS</td>
</tr>
</tbody>
</table>

*Abbreviations: *P* = significance of difference between mean values and mean of preceding group; NS = not significant (*P* > 0.05). Values are mean ± SE.

*Although not significantly different from preceding value, significantly different from control value (*P* < 0.025). Number in parentheses is number of experiments.
Data on myocardial nucleoside content during coronary occlusion, plotted as a function of the number of beats during coronary occlusion. There are fewer inosine and hypoxanthine data because these compounds were not assayed individually. The accumulation of adenosine appears to be a linear function of the number of beats during coronary occlusion. Inosine accumulation appears to be similar to that of adenosine, but hypoxanthine content appears to remain constant.

the two nucleosides in control hearts was 1.07 nmole/g, the sum of the two determined separately in experiments with 15 seconds of coronary occlusion was 1.98 nmole/g. The myocardial content of hypoxanthine in those experiments in which this nucleoside was assayed separately did not tend to increase, while that of inosine did increase with lengthening duration of occlusion. The data in Table 1 suggest that there may be a slight increase in the rate of nucleoside production between the tenth and fifteenth seconds of coronary occlusion. This is probably due to higher heart rates in this group of animals, however, for when adenosine and inosine data are plotted as a function of the number of heart beats occurring between the onset of coronary occlusion and the time of biopsy, the increase in the content of these nucleosides appears to be linear (Fig. 2). There did not appear to be an increase in hypoxanthine content of these hearts during coronary occlusion.

Discussion

The estimates of myocardial nucleoside content obtained in the present study were made possible by the use of a spectrophotometer capable of measuring absorbancy changes of less than $10^{-4} \text{cm}^{-1}$ with a precision on the order of $10^{-5} \text{cm}^{-1}$. This instrument had an additional advantage in that it is designed to measure these small absorbancy changes in solutions having high background absorption, thus making it possible to assay nucleosides in crude tissue extracts. The sensitivity of the assay for adenosine was enhanced by converting the adenosine to uric acid. The absorbancy change at 293 nm for uric acid production is about 1.5 times the decrease in absorbancy at 265 nm when an equivalent amount of adenosine is deaminated to inosine. Depending upon the amount of tissue obtained, these modifications have improved the sensitivity of the assay by as much as 200-fold.

The reproducibility of the tissue processing and chemical assay techniques appear to be satisfactory. Nearly half the tissue assays agreed to within 0.1 nmole/g. Instrument noise level was less than $2 \times 10^{-4} \text{cm}^{-1}$ which would account for a variability of not more than $\pm 0.02$ nmole/g in tissue content. Ab-
sorbancy changes were estimated to the nearest 0.5% of full scale on the recorder chart, which contributed an additional variability of as much as ±0.02 nmole/g in tissue nucleoside content. A troublesome and frequent source of error was distortion of the absorbancy trace by the passage of motes of dust through the optical path during the course of assay. Even though these were almost invisible and were partially compensated for by the use of a double-beam instrument, the high sensitivity of the instrument magnified their influence. This was usually responsible for the larger differences between duplicates seen in some assays. Other sources of error such as those due to tissue weighing, pipetting, and to condensation of moisture on frozen tissue, etc., were not quantified.

Rubio et al. (6) have used levels of adenosine in coronary sinus blood collected during reactive hyperemia to arrive at an estimate of the adenosine content of the normally oxygenated heart, 10^{-14} mol/g, which is 30 times lower than the average value obtained by direct analysis in the present study. A subsequent estimate by the same authors of tissue adenosine content based on the adenosine concentration of pericardial perfusates gave a considerably higher value, 22 \times 10^{-14} \text{mol/g} (9). While the reason for this discrepancy is not clear, the results of the present study suggest that the latter value may be more nearly correct.

In the present study, myocardial adenosine content rose during coronary occlusion, and the increase appeared to be related more closely to the number of beats occurring during occlusion than to its duration. This evidence suggests that adenosine production is somehow linked to the metabolism of the heart, as heart rate is an important determinant of myocardial oxygen consumption (10). The myocardial content of the sum of inosine and hypoxanthine also rose, though at a slower rate. The limited data on individual nucleoside content suggests that the increase in the sum of the two compounds was largely due to an increase in inosine content.

The rise in adenosine content of the left ventricle during coronary artery occlusion found in this study is consistent with the proposal of Rubio et al. (6) that adenosine may be responsible for the vasodilation of myocardial reactive hyperemia.

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References

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