Cardiac nucleotides in hypoxia: possible role in regulation of coronary blood flow

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BERNE, ROBERT M. Cardiac nucleotides in hypoxia: possible role in regulation of coronary blood flow. Am. J. Physiol. 204(2): 317-322. 1963.—Experiments were performed on isolated cat hearts perfused with Tyrode’s solution and intact hearts of open-chest dogs. Cardiac hypoxia resulted in a decrease in coronary vascular resistance and a release of significant amounts of inosine and hypoxanthine from the myocardium. From 3 to 27 times more inosine and hypoxanthine were released from the heart during myocardial hypoxia than were required to double the coronary blood flow when infused as adenosine into the left coronary artery. Based on the assumption that with hypoxia the nucleotide derivatives leave the myocardial cell as adenosine, an hypothesis is proposed for the metabolic regulation of coronary blood flow.

At a constant level of energy expenditure by the myocardium, coronary blood flow is relatively stable in the face of alterations in perfusion pressure (1, 2). The nature of the mechanism responsible for this phenomenon, known as autoregulation, remains obscure. The cardiac nerves are apparently not involved, since autoregulation is readily observed in the denervated heart. However, the occurrence of reactive hyperemia in the heart and the striking parallelism between coronary blood flow and the metabolic activity of the myocardium have directed attention to products of metabolism as possible mediators. To date, none of the known metabolites or vasoactive constituents of cardiac muscle have been shown to be released from the myocardium in concentrations sufficient to account for the coronary vasodilation observed with hypoxia or augmented cardiac metabolism (3). Furthermore, reoxygenated coronary sinus blood collected from hearts subjected to hypoxia or ischemia failed to produce changes in vascular resistance when infused into test coronary vessels (4). This observation could mean that if a vasoactive substance is released from the myocardium during hypoxia it is rapidly inactivated and/or greatly diluted by the blood.

Adenosine may conceivably be the vasoactive substance since: 1) it is a potent coronary vasodilator (5); 2) it is rapidly inactivated by adenosine deaminase in blood and tissues (6, 7); 3) it can rapidly penetrate the myocardial cell membrane (7); and 4) there is a large potential source of adenosine in the form of adenine nucleotide in the myocardium. Analysis of nucleotide derivatives in perfusates and coronary sinus blood was undertaken to test the hypothesis that the release of adenosine from the myocardium is responsible for the coronary vasodilation observed in cardiac hypoxia.

METHODS

Cat heart experiments. Cats were struck a sharp blow on the head and the hearts of the unconscious animals were quickly excised, rinsed with warm Tyrode’s solution, and perfused with oxygenated Tyrode’s solution in the manner originally described by Langendorff (8). The perfusion fluid was equilibrated with a gas mixture of 95% O2 and 5% CO2 and introduced into the aorta at a pressure of 40 cm of water. After all residual blood was flushed out of the heart, a control period was begun in which 200 ml of oxygenated Tyrode’s solution was recycled through the heart for 15 min and then collected in iced flasks. The heart was then made anoxic by changing the perfusion fluid to Tyrode’s solution with the addition of 5% N2 and 5% CO2.

Two hundred milliliters of this anoxic perfusion fluid was recycled through the heart for 15 min and collected in iced flasks. The perfusion fluid was equilibrated with oxygenated Tyrode’s solution and the perfusate collected. The perfusates were then treated with perchloric acid for protein precipitation and the nucleotide derivatives adsorbed on charcoal, eluted, separated on Dowex-1-chloride columns and assayed enzymatically as previously described (7).

Dog heart experiments. Dogs were anesthetized with intravenously administered sodium pentobarbital (30 mg/kg), placed on artificial respiration, and thoracotomized in the fourth left intercostal space. After the ad-
ministration of heparin, the left coronary artery was can-
nulated with an Eckstein cannula and the coronary sinus can-
nulated with a modified Marwazid cannula. The left
coronary artery was perfused with blood from the left carotid
tube and the coronary sinus outflow was di-
dicted to the left jugular vein. Coronary blood flow was
measured in a rotameter and mean left coronary pcr-
fusion pressure with a mercury manometer. During con-
trol periods the animal was inspired with room air,
whereas during the experimental periods partial as-
phyxia was produced by greatly reducing the respira-
tory minute volume. Approximately 30 ml of coronary
arterial and coronary sinus blood were simultaneously
obtained from the tubing of the respective cannulae. In
experimental periods, when asphyxia was well estab-
lished, coronary blood flow was reduced to control levels
by an adjustment of a screw clamp on the coronary
artery inflow tubing before blood samples were drawn.
The blood was immediately chilled and the red cells
sedimented in a refrigerated centrifuge. Fifteen to
twenty milligrams of acid-washed, activated wood char-
cel (Darco) was added to each plasma sample (ca.
20 ml) and the suspension shaken in a cold room for 60 min.

After centrifugation, the plasma was discarded and the
sedimented charcoal was transferred with 15 ml of water
to a column 1 cm in diameter containing 150 mg of
washed Celite packed over a small glass-wool plug. Fifty
per cent ethanol at pH 10 was put through the column
until the eluate read zero against the eluting solution in
the Beckman model DU spectrophotometer at wave
lengths of 249 and 260 nm. The eluate samples were
recovered upon concentration under a filter air blower
bottom of the jar. After approximately 50 hr the strips
were removed and air dried. The UV-absorbing spots
were cut out, placed between glass slides and the ma-
terial eluted with water in a chromatography jar satu-
rated with water vapor. All UV-absorbing material was
eluted from the small strips in 1-3 ml water in about 20
hr. Volumes were adjusted to 2.1 ml by evaporation at
40°C by the addition of water. Aliquots of this solution
were then analyzed for their inosine and hypoxanthine
content by the enzymatic methods of Kalckar (9) using
microcuvettes in the Beckman model DU spectro-
photometer. All determinations were made in duplicate.

RESULTS

Cat heart experiments. Adenine nucleotides and adenosine
were not present in the perfusates of hearts perfused with
oxygenated or deoxygenated Tyrode's solution. It is
quite possible that adenosine was released from the per-
 fused heart but was degraded to inosine and/or hypo-
xanthine before the addition of perchloric acid to the
perfusate. Previous studies (7) have shown that adenosine
deaminase and nucleoside phosphorylase, which convert
adenosine to inosine and hypoxanthine, are leached out of
hearts perfused with oxygenated Tyrode's solution.

Replacement of oxygenated Tyrode's solution with
Tyrode's solution equilibrated with nitrogen resulted in
the appearance of hypoxanthine and inosine in the per-
 fusate (Table 1). Reintroduction of oxygenated perfusion
fluid produced a decrease in the amounts of these com-
pounds released from the heart. Although the absolute
values for the hypoxanthine and inosine content of the
perfusates are large they represent only about 0.3-0.4%
of the total adenine nucleotide content of the myo-
cardium. Coronary vasodilation and a reduction in heart
rate were associated with the anoxia-induced appearance
of inosine and hypoxanthine. The bradycardia is indica-
tive of the severe degree of anoxia produced in these
experiments.

Dog heart experiments. The isolated Tyrode-perfused
nonworking cat heart was employed in the initial studies
because it is a simple preparation free of possible con-
taminating nucleotide derivatives from erythrocytes and
one which can be subjected to complete anoxia. Gross
changes observed with such a preparation may be indica-
tive of normal phenomena but, because of the unphysio-
logical nature of the preparation, extrapolation to
normal heart muscle could be misleading. Therefore, the
next series of experiments was carried out on the intact
working heart in the open-chest dog in order to deter-
mine whether similar observations could be made under
more physiological conditions. Before undertaking
animal experiments, it was considered desirable to
demonstrate to what extent and with what degree of
accuracy nucleotide derivatives could be recovered from
dog plasma.

Separation of the nucleotide derivatives was accom-
cplished by paper chromatography, since the use of
columns resulted in poor recoveries when small amounts

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Coronary Flow, ml/min</th>
<th>Heart Rate, beats/min</th>
<th>Hypoxanthine, μg/20 ml Perfusate</th>
<th>Inosine, μg/20 ml Perfusate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>17</td>
<td>74</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Anoxia</td>
<td>20</td>
<td>29</td>
<td>0.166</td>
<td>0.233</td>
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<tr>
<td>Recovery</td>
<td>9</td>
<td>50</td>
<td>0.088</td>
<td>0.210</td>
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<tr>
<td>Control</td>
<td>14</td>
<td>128</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Anoxia</td>
<td>21</td>
<td>88</td>
<td>0.094</td>
<td>0.250</td>
</tr>
<tr>
<td>Recovery</td>
<td>14</td>
<td>162</td>
<td>0.021</td>
<td>0.062</td>
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<tr>
<td>Control</td>
<td>15</td>
<td>120</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Anoxia</td>
<td>30</td>
<td>77</td>
<td>0.023</td>
<td>0.191</td>
</tr>
<tr>
<td>Recovery</td>
<td>17</td>
<td>108</td>
<td>0.028</td>
<td>0.088</td>
</tr>
</tbody>
</table>
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made detection of these nucleotide derivatives impossible. In these experiments revealed insignificant quantities of material were used. However, it was necessary to have approximately 10 μmole or more per spot in order to be able to delineate the area on the paper under UV light with relative ease. In these amounts, recoveries from the paper were quantitative. Percentage recoveries of inosine and hypoxanthine from dog plasma were determined at plasma concentrations of 1-6 μmole/ml plasma in 28 trials using 6 different plasma samples. At these concentrations the per cent recovery of added hypoxanthine was 80.9, with a standard deviation of 4.4-19.2. A second period of asphyxia, more severe than the first, was associated with an increase in the concentration of hypoxanthine in the coronary sinus blood and the appearance of significant amounts of inosine. In the remaining experiments presented in Table 2, coronary blood flow was adjusted to approximately control levels during the asphyxia period by means of a screw clamp on the arterial inflow tubing. As can be seen from the reduction in perfusion pressure required to maintain a relatively constant coronary blood flow, vascular resistance was significantly reduced by the partial asphyxia. On release of the constriction and restoration of perfusion pressure to approximately control levels, coronary blood flow showed a large overshoot above control.

The data from the one experiment in which significant quantities of hypoxanthine were present in coronary sinus blood (with asphyxia), despite a twofold increase in coronary blood flow, are shown in Table 2 (exp. 3). A second period of asphyxia, more severe than the first, was associated with an increase in the concentration of hypoxanthine in the coronary sinus blood and the appearance of significant amounts of inosine. In the remaining experiments presented in Table 2, coronary blood flow was adjusted to approximately control levels during the asphyxia period by means of a screw clamp on the arterial inflow tubing. As can be seen from the reduction in perfusion pressure required to maintain a relatively constant coronary blood flow, vascular resistance was significantly reduced by the partial asphyxia. On release of the constriction and restoration of perfusion pressure to approximately control levels, coronary blood flow showed a large overshoot above control.

During each of the hypoxic periods of the last four experiments in Table 2, the coronary sinus plasma fraction contained 4.4–19.2 μmole hypoxanthine and 1.0–15.8 μmole inosine/ml plasma, whereas simultaneously drawn arterial blood samples contained 0.0–0.4 μmole hypoxanthine/ml plasma and no inosine. In control and recovery arterial and venous plasma samples inosine was absent and the maximum concentration of hypoxanthine found was 0.7 μmole/ml.

An approximation of the amounts of nucleotide derivatives released per minute from the heart during the periods of partial asphyxia is presented in Table 2. These estimates are products of the coronary blood flow in milliliters per gram wet weight of heart and the sum of the arteriovenous differences for inosine and hypoxanthine. The values ranged from 2.7 to 21.3 μmole/min g heart.

Glutamic oxalacetic transaminase determinations were carried out on control and experimental arterial and venous blood samples from 6 hearts. On the average, the values were found to be approximately 10.2 ± 0.33.3 on September 6, 2017.
were 0.84 and 0.80 for experiments g and h with the rate of release in asphyxia (Table 3). This amount of adenosine produced a two- to three-fold increase in coronary blood flow which was due primarily to approximately control levels by compression of the coronary artery at a point proximal to the rotameter (Table 3). This amount of adenosine produced a twofold increase in coronary blood flow which was returned to approximately control levels by compression of the inflow tubing prior to simultaneous arterial and venous sampling. Complete mixing of the infused adenosine with the arterial blood had occurred by the time the blood reached the point where arterial samples were drawn. Analysis of coronary arterial and venous blood samples revealed the absence of hypoxanthine and the presence of inosine in the arterial blood. In these experiments any adenosine would appear as inosine, since no attempt was made to separate the two nucleosides. No hypoxanthine was found. A second problem is the uptake and metabolism of nucleotides. The experiments on the intracoronary infusion of adenosine (Table 3) indicate that approximately one-half of the expected concentration of inosine in the arterial blood samples in experiments 6–g (Table 2) was 5.3 and 3.7 μmoles/ml plasma, respectively. The amounts recovered were approximately one-half of the expected concentration, namely 2.9 and 1.9 μmoles/ml plasma. When expressed as millimicromoles reaching one gram of myocardium per minute, for purposes of comparison with the rate of release in asphyxia (Table 2), the values were 0.84 and 0.80 for experiments g and h, respectively.

### Table 3. Effect of intracoronary infusion of adenosine on coronary blood flow

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Perfusion Pressure, mm Hg</th>
<th>Coronary Blood Flow, ml/min</th>
<th>Inosine, μmoles/ml Plasma</th>
<th>Inosine, μmoles/min/g Heart</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>84</td>
<td>59</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Adenosine†</td>
<td>76</td>
<td>124</td>
<td>2.9</td>
<td>0.84</td>
</tr>
<tr>
<td>Adenosine with constriction of tubing</td>
<td>55</td>
<td>47</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Adenosine with release of constriction</td>
<td>82</td>
<td>105</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Recovery</td>
<td>0</td>
<td>64</td>
<td>0</td>
<td>0</td>
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</tbody>
</table>

**Control**

<table>
<thead>
<tr>
<th>Experiment 6 (162)</th>
<th>Arterial</th>
<th>Venous</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenosine†</td>
<td>92</td>
<td>71</td>
</tr>
<tr>
<td>Adenosine with constriction of tubing</td>
<td>51</td>
<td>71</td>
</tr>
<tr>
<td>Adenosine with release of constriction</td>
<td>80</td>
<td>158</td>
</tr>
<tr>
<td>Recovery</td>
<td>88</td>
<td>68</td>
</tr>
</tbody>
</table>

**Adenosine**

<table>
<thead>
<tr>
<th>Experiment 7 (163)</th>
<th>Coronary Blood Flow, ml/min</th>
<th>Inosine, μmoles/ml Plasma</th>
<th>Inosine, μmoles/min/g Heart</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>88</td>
<td>59</td>
<td>0</td>
</tr>
<tr>
<td>Adenosine†</td>
<td>76</td>
<td>124</td>
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<td>105</td>
<td>0</td>
</tr>
<tr>
<td>Recovery</td>
<td>0</td>
<td>64</td>
<td>0</td>
</tr>
</tbody>
</table>

**Adenosine**

**Inosine**, * Includes adenosine since no attempt was made to separate the two nucleosides. * Hypoxanthine was found. † Adenosine dissolved in 0.9% NaCl infused at 0.25 μmoles/min into left coronary artery.

Numbers in parentheses refer to wet weight of perfused myocardium.

### Discussion

With hypoxia, inosine and hypoxanthine appeared in the perfusate of the isolated cat heart and in the coronary sinus blood of the intact, working dog heart. Neither of these adenine nucleotide derivatives was present in significant amounts in arterial blood of the hypoxic dog or in arterial or cardiac venous blood of the normally ventilated animal. These observations indicate that the inosine and hypoxanthine originated in the myocardium and that the partial asphyxia in the dog did not produce a release of such compounds from other body tissues in quantities sufficient to be detected in the arterial blood. Because of its simplicity the Langendorff preparation was ideally suited for preliminary studies. However, the release of inosine and hypoxanthine from the anoxic cat heart may not be physiologically significant, since the heart is perfused with salt solution and is somewhat hypoxic even during control periods, as suggested by the leakage of enzymes into the perfusion fluid (7). In contrast, the hypoxic dog heart is capable of performing work and shows no impairment of the integrity of the cell membranes, as evidenced by the absence of transaminase or adenosine deaminase in coronary sinus blood. However, the presence of erythrocytes in the dog heart experiments poses several problems. The erythrocytes are rich in ATP and thus are a potential source of contaminating adenine nucleotide derivatives. Therefore, it is necessary to exercise considerable care in the collection and handling of the blood samples to avoid red cell lysis. A second problem is the uptake and metabolism of nucleosides by the red cells. Inosine and adenosine readily enter red cells where the ribose moiety is metabolized and the purine can be utilized in the synthesis of nucleotides. The experiments on the intracoronary infusion of adenosine (Table 3) indicate that approximately one half of the nucleoside disappeared between the point of infusion and the point of sampling (transit time between these points being about 15 sec). Presumably the lost nucleoside was in the erythrocytes. A third problem related to the use of blood as a perfusion medium is the presence of adenosine deaminase and nucleoside phosphohydrolase in erythrocytes. These enzymes rapidly degrade adenosine and thereby make accurate calculation of the effective concentration of adenosine at its site of action virtually impossible.

The amount of the nucleotide derivatives released per minute from the hypoxic dog heart constitutes a small fraction (approx. $1/20$ to $1/2000$) of the available adenine nucleotide, in contrast to complete anoxia in which the total adenine nucleotide content of the intact heart is reduced to about 60% of normal in a period of 45 min (10). However, if the hypoxic period were to persist for more
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In arterial blood, adenosine is released from the myocardium and diffuses into the coronary vessels. The adenosine elicits a twofold increment in coronary blood flow when infused as a vasodilator 

Furthermore, when the infused adenosine (Table 3) and the nucleotide derivatives released from the heart during moderate to severe asphyxia (Table 2) are compared on the basis of equal amounts of myocardium perfused per unit time, one finds that from 3 to 27 times more inosine and hypoxanthine are released from the heart during myocardial hypoxia than are required to double the coronary blood flow when infused as adenosine into the left coronary artery. These calculations indicate that if the nucleotide derivatives are released from the myocardium as adenosine, only a small fraction of the amount recovered in the present study is necessary to produce a marked reduction in coronary resistance. Therefore it is possible that less drastic and more physiological stimuli than those employed in these experiments could bring about the release of vasoactive amounts of adenosine from the myocardium which could not be detected in coronary sinus blood by the methods presently available.

In none of the cat or dog experiments were adenosine or the adenosine nucleotides detected in the heart effluents. There is no good evidence that the phosphorylated adenosine compounds can traverse the intact cell membrane, although adenosine has been shown to enter myocardial cells with relative ease (7). Adenosine and the adenosine nucleotides are potent vasodilators, whereas inosine and hypoxanthine are virtually ineffective in altering vascular resistance. To be of physiological significance in the regulation of coronary blood flow it must be demonstrated that the inosine and hypoxanthine leave the myocardial cell in a form that has vasodilator activity. At present it has not been possible to show that any of the recoverable nucleoside derivatives cross the myocardial cell membrane as adenosine. In the cat heart experiments adenosine deaminase and nucleoside phosphorylase are leached out of the perfused heart and any adenosine released into the perfusate would be converted to inosine and hypoxanthine before in vitro inactivation of these enzymes could be accomplished (7). In the dogheart experiments the time required to collect the coronary sinus blood and to separate cells and plasma is sufficient for complete destruction of any adenosine which might have been present.

Studies by Angkapindu, Stafford, and Thorp (11) on aqueous extracts of guinea pig heart suggest that dephosphorylation of adenylic acid (AMP) precedes deamination. If this is true in the intact heart it is conceivable that the inosine found in the heart effluents is in some measure derived directly from adenosine by deamination and not by the dephosphorylation of inosinic acid. Based on the assumption that a significant amount of the adenosine formed from AMP is deaminated outside of the myocardial cell, the following hypothesis for the metabolic regulation of coronary blood flow is proposed (Fig. 1). In this scheme the key factor is the myocardial oxygen tension. Reduction in myocardial oxygen tension by hypoxemia, decreased coronary blood flow, or increased oxygen utilization by the myocardial cell leads to the breakdown of heart muscle adenine nucleotides to adenosine. The adenosine diffuses out of the cell and reaches the coronary arterioles via the interstitial fluid and produces arteriolar dilation. The resultant increase in coronary blood flow elevates myocardial oxygen tension, thereby reducing the rate of degradation of adenosine nucleotides, and decreases the interstitial fluid concentration of adenosine by washout and enzymatic destruction. This feedback mechanism serves to adjust coronary blood flow to meet the new metabolic requirements and a new steady state is reached. Restoration of normal myocardial oxygen tension prevents the degradation of adenosine nucleotides and the formation of
adenosine, and permits the intrinsic vascular tone to reduce coronary blood flow to control levels.

REFERENCES


The author wishes to express his gratitude for the excellent technical assistance of Miss M. Hadady.