Release of Adenosine by the Normal Myocardium in Dogs and Its Relationship to the Regulation of Coronary Resistance

By Rafael Rubio, Ph.D., and Robert M. Berne, M.D.

ABSTRACT

The evidence supporting the hypothesis that adenosine is the mediator of metabolic regulation of coronary blood flow was obtained from experiments characterized by myocardial hypoxia. If adenosine serves the role of physiological regulator of coronary blood flow, it must also be released by the normal heart. Experiments designed to study this question were performed on 15 open-chest dogs in which adenosine was sought in perfusates of the epicardial surface of the well-oxygenated heart. The pericardial space was perfused with warm (37°C) Tyrode's or Krebs-Henseleit solutions (400 to 1200 ml over 1 to 3 hours), and the perfusates were analyzed for adenosine. With a normal myocardial oxygen supply, adenosine was present in the perfusates in a concentration of 3.1 ± 0.5 × 10^-8 M. Partial asphyxia, induced by reducing pulmonary ventilation, significantly (P < 0.02) increased the adenosine concentration of the perfusates to 5.4 ± 0.8 × 10^-8 M. In four dogs the normal pericardial fluid was found to contain adenosine in a concentration of 10.9 ± 2.9 × 10^-7 M, which probably represents the basal extracellular adenosine concentration in the myocardium. The results indicate that the normal myocardial cells release adenosine continuously into the surrounding interstitial fluid, and it is suggested that the level of the interstitial fluid concentration of adenosine probably regulates coronary blood flow to maintain the oxygen balance of the myocardium.

ADDITIONAL KEY WORDS coronary blood flow myocardial metabolites myocardial nucleotides autoregulation of coronary blood flow adenine nucleotide degradation in heart vascular smooth muscle relaxation superfusion of cardiac surface control of arterioles in the heart myocardial oxygen balance

Coronary blood flow normally correlates well with myocardial oxygen balance (1-5), and it has been suggested that the tone of the coronary vascular bed is significantly affected by the metabolism of the myocardial cells (2, 6-8). However, the precise link between coronary vascular tone and the metabolic activity of the myocardium is unknown (9). One possible mediator of metabolic regulation of coronary blood flow is adenosine (10), a powerful coronary vasodilator. The adenosine hypothesis proposes that adenosine is continuously released by the myocardial cells into the interstitial fluid, and its release is enhanced in response to either an increase in cardiac oxygen consumption or a reduction in the arterial oxygen supply. An increase in the concentration of adenosine in the interstitial fluid would cause the coronary resistance vessels to dilate and the coronary blood flow to increase to the point at which the original myocardial oxygen balance would be restored. In this fashion, adenosine may function as a signal to indicate the amount of oxygen required by the myocardium.

The adenosine hypothesis is based on data obtained under experimental conditions of myocardial hypoxia, and hence it is inconclu-
sive as to its relevance to the normal physiological regulation of coronary blood flow. Recently, it has been possible to isolate adenosine from coronary sinus blood during the periods of reactive hyperemia following brief periods of coronary occlusion (11). The concentration of adenosine that was calculated to be present in the myocardial interstitial fluid during reactive hyperemia exceeds the concentration required to elicit maximal coronary dilation (11).

Whether adenosine is involved in the regulation of coronary blood flow in the absence of myocardial hypoxia has not been evaluated because of the difficulties in detecting the minute concentrations of the nucleoside required to induce small degrees of coronary dilation (11). To accept the applicability of the adenosine hypothesis to the physiological regulation of coronary blood flow, it must be demonstrated that: (1) there is a continuous release of adenosine by the normal myocardial cells into the interstitial fluid, (2) the actual product released is not an adenosine precursor but is adenosine itself, and (3) there is a precise quantitative cause-and-effect relationship between the interstitial fluid concentration of adenosine and the diameter of the coronary resistance vessels. The present report represents an attempt to satisfy the first criterion.

Methods

Dogs weighing between 16 and 20 kg were anesthetized with pentobarbital, 30 mg/kg, and after starting artificial respiration their chests were opened by a midsternal incision. The fat and small vessels surrounding the pericardium were removed, and one or two silastic tubes (4 mm diameter) were tied into the pericardial sac for superfusion of the surface of the heart. When two tubes were used, the opening of one was placed anteriorly near the apex of the heart and the second tube was placed posteriorly at the base of the heart. The apical tube served as an inlet and the basal tube as an outlet for the perfusing fluid. When just one tube was used, its opening was placed at the base of the heart, and it served as an inlet and outlet for fluid left in contact with the epicardial surface for varying periods of time. Either Tyrode’s or Krebs-Henseleit solutions (37°C, pH 7.4, and containing 5% dextran) served as the perfusion fluid. The solutions were equilibrated with mixtures of either 95% O₂ and 5% CO₂ or 10% O₂, 85% N₂, and 5% CO₂.

In some experiments, heparin was administered and an extracorporeal dialyzing system was placed in series with a femoral artery. The cellulose dialysis tubing through which the blood flowed had a mean pore radius of 24 Å which permitted solutes below a molecular weight of 30,000 to move freely across the membrane. The dialysis tube was 3 cm in diameter, 24 to 25 cm in length, and had a surface area of 230 cm². This surface area is approximately equal to that of the epicardium, estimated by dividing the mean weight of a pericardial sac (averaged from six different animals) by the mean weight of 1 cm² of sac. The perfusion fluid used for dialysis of the femoral arterial blood was the same with respect to composition and volume as that used for superfusion of the epicardial surface of the heart.

The pericardial perfusates and blood dialysates were collected by two different procedures. In experiments 1 through 8 the perfusion or dialyzing fluid was infused continuously at a rate between 5 and 8 ml/min, whereas in experiments 9 through 15, 75 to 85 ml of fluid was introduced into the pericardial sac or dialyzing system and left there for specified periods of time. The volume used was determined by the size of the heart. The fluid in the pericardial sac and outside the dialysis tubing was simultaneously replaced by equal volumes of fresh fluid every 10 minutes. In the case of the blood dialysates, the fluid was stirred by rapid withdrawal and reinjection with a large syringe every 2 or 3 minutes. Each aliquot of pericardial and dialysis fluid was immediately placed in boiling water for 10 minutes to destroy any enzymatic activity. They were then pooled and processed for adenosine determination as previously described (11). The total volume of fluid collected in each experiment was between 400 and 1,200 ml and was collected over a period of 1 to 3 hours.

Protocol 1.—In the first series of experiments (1 through 7) samples of pericardial perfusates were collected under control conditions and during partial asphyxia. Asphyxia was produced by interrupting the artificial respiration every fifth minute for 1 minute. The perfusion fluid used during the periods of partial asphyxia was equilibrated with 10% O₂, 5% CO₂, and 85% N₂.

Protocols 2 and 3.—In the second series of experiments, two different protocols were followed. In one group, (8 through 11, protocol 2), the control period of pericardial perfusion was followed by a period of asphyxia, and that, in turn, by a recovery period. In all of these experiments, a blood dialysate sample was obtained simultaneously with each pericardial

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perfusate sample. In the second group of experiments (12 through 15, protocol 3) 75 to 85 ml of perfusion fluid was introduced into the pericardial sac and removed 2.5 minutes later and kept at 37°C in a water bath. This sample was designated “control.” Immediately after collection of the control sample, artificial respiration was stopped for a period of 75 seconds, and 15 seconds after the start of asphyxia an equal volume of fresh perfusion fluid was injected into the pericardial sac. It was removed 2.5 minutes later and kept at 37°C; it was designated “asphyxia.” Immediately after collection of the first asphyxia sample, the first control sample was reintroduced into the pericardial sac and the cycle repeated. Each control and asphyxia sample was exposed to the heart four times for a total contact time with the epicardial surface of 10 minutes. After the fourth exposure, each sample was immersed in boiling water for 10 minutes. The cycle was then repeated with an equal volume of fresh perfusion fluid. From 24 to 32 cycles were carried out in each experiment. All control samples were pooled, as were all asphyxia samples. The blood dialysates were cycled in a similar manner, but the control samples were pooled with the asphyxia samples.

The volume (V) of the pericardial fluid normally existing in the pericardial sac was also measured in four dogs and its adenosine content determined. This volume was usually very small and since it is impossible to withdraw all of it for quantitation, its volume was estimated by the dilution of an inulin solution introduced into the sac. The following procedure was used. Five milliliters of perfusion fluid was introduced into the pericardial sac (total volume of sac = 5 + V ml), and 1 minute later 0.5 ml was removed and used in the inulin blank determination. Immediately thereafter, 1 ml of inulin solution (C1 = 2 mg/ml) was introduced into the pericardial sac (total volume = 5.5 + V ml), and 30 seconds later 0.5 ml of pericardial fluid was removed for measurement of the inulin concentration (C2). Then, 80 ml of perfusion fluid was introduced into the sac, and 1 minute later all the fluid that could be withdrawn from the sac was removed and another 80 ml of fresh perfusion fluid was introduced. The second 80 ml was removed 1 minute later, and the two pericardial washes were pooled. The combined sample was analyzed for adenosine, and the amount found was taken to represent the total present in the undiluted pericardial fluid. The inulin determination was made by the method of Roe et al. (12), and the pericardial fluid volume was determined by applying the following formula: C1 × 1 ml = C2 (5.5 + V) and solving for V.

### TABLE 1

<table>
<thead>
<tr>
<th>Expt. no.</th>
<th>Control Pericardial perfusate (X 10^{-8} M)</th>
<th>Asphyxia* Pericardial perfusate</th>
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<th>Recovery Pericardial perfusate</th>
<th>Blood dialysate</th>
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* Respirator was interrupted for 1 minute every 5 minutes for protocols 1 and 2, and for 75 seconds every 5 minutes for protocol 3. † The control samples were pooled with these.
TABLE 2
Adenosine in Pericardial Perfusates and Arterial Blood Dialysates for All Experiments

<table>
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<th>No. expts.</th>
<th>No. determinations</th>
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<tr>
<td><strong>Blood Dialysates</strong></td>
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<td>0.6 ± 0.2 (sk)</td>
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</tbody>
</table>

* The difference between the adenosine concentration in the pericardial perfusate of the control and asphyxia samples is significant (P < 0.02).

Results

ADENOSINE IN PERICARDIAL PERFUSATES

Adenosine was found in each of 31 samples of pericardial perfusate obtained in 15 experiments. Table 1 (protocol 1) presents the results of the first seven experiments. In experiments 4 through 7, the concentration of adenosine was found to be increased in the pericardial perfusates collected during asphyxia following a control period.

It seemed possible that the adenosine in the pericardial perfusates may have originated in the coronary blood and not in the myocardium, even though we had previously been unable to find adenosine in plasma samples of arterial blood. Even with arterial blood concentrations of adenosine below detectable levels, the large volume of perfusion fluid could pick up enough of the nucleoside by dialysis of the coronary blood on or near the surface of the heart to yield measurable quantities. Therefore, the arterial blood contribution of adenosine to the pericardial perfusates was determined, and the results are reported in Table 1 (protocols 2 and 3). Most of the blood dialysates showed the presence of adenosine, but the concentration was lower than that of the corresponding pericardial perfusates. Furthermore, adenosine was absent in some of the samples.

To avoid possible artifacts introduced by the order of sampling, the second series of experiments was undertaken according to protocols 2 and 3. These experiments eliminate the possibility of sampling as the cause of the increased adenosine concentration in the pericardial perfusates collected during the asphyxia period. The results are presented in Table 1. Although the differences were small, the adenosine concentrations of the pericardial perfusates in the asphyxia samples were, in general, higher than the corresponding control and recovery samples. The only exceptions were experiments 10 and 13. Table 2 summarizes all the data on the adenosine concentration of pericardial perfusates for the experiments presented in Table 1, as well as all the data on the adenosine concentration of the blood dialysates. The adenosine concentration of the control pericardial perfusate was fivefold greater than that of the blood dialysates (3.1 vs. 0.6).

ADENOSINE IN PERICARDIAL FLUID

The pericardial fluid volume, the total amount and concentration of adenosine present in pericardial fluid, and the calculated amount of adenosine per gram of myocardium are given in Table 3. The calculated total amount of adenosine in normal pericardial fluid is very small, and its precise quantitation...
is beyond the limits of our methods. Nevertheless, the approximations reported here are reasonable since the enzymatic assays can detect as low as 1 μmole and can quantitate 3 μmoles with precision. For calculation of the amount of adenosine per gram of myocardium, it was assumed that the adenosine is restricted to the extracellular space (see discussion) and that the extracellular fluid volume comprises 0.2 ml/g of wet myocardium (13-15). Hence, 10.9 × 10⁻¹⁰ moles/ml of interstitial fluid × 0.2 = 2.2 × 10⁻¹⁰ moles/g of myocardium.

**Discussion**

The fact that adenosine is found in pericardial fluid and pericardial perfusates demonstrates that the normal myocardium releases adenosine into the surrounding fluid. This release is accelerated by asphyxia. The possibility of pericardial adenosine originating in blood can be eliminated because the concentration of adenosine in blood dialysates was consistently lower than that of the corresponding pericardial perfusates. The normal pericardial fluid is probably representative of the myocardial interstitial fluid (16). Therefore, the concentration of adenosine in normal pericardial fluid should represent the basal extracellular adenosine concentration in the myocardium. This, of course, only holds true for normal pericardial fluid collected under steady-state conditions. With hypoxia or during pericardial perfusion there is in all likelihood a steep gradient in the adenosine concentration from interstitial fluid to pericardial fluid (or perfusate), making calculations of myocardial adenosine concentrations and any attempts at correlation of adenosine release with coronary blood flow meaningless. For this reason coronary flow was not measured in the present study.

The restriction of adenosine to the interstitial spaces is a reasonable assumption based on the cellular localization and properties of the enzymes 5'-nucleotidase, adenylic acid deaminase (AMP-deaminase), and adenosine deaminase, as revealed by histochemical and cell fractionation studies (17-19). The localization of these enzymes is illustrated in Figure 1 which represents a myocardial cell surrounded by interstitial fluid, a capillary, and an arteriole. AMP is hydrolyzed to adenosine by the 5'-nucleotidase which, according to histochemical (19) and cell fractionation studies (17, 18) is primarily bound to the cell membrane. However, some intracellular AMP is evidently deaminated to inosinic acid (20) by the AMP-deaminase (17, 18). Hence, there is probably no free adenosine within the myocardial cell because of the high protoplasmic concentration of adenosine deaminase (17, 18).

Some of the adenosine moving back from the interstitial fluid into the myocardial cell is rephosphorylated to AMP (21) by action of adenosine kinase (22). Phosphorylation of adenosine occurs by some unknown mechanism which circumvents the action of the adenosine deaminase, as indicated by the fact that 14C-adenosine infused into the coronary arteries at low concentrations is readily incorporated into the adenine nucleotide pool with a negligible degree of deamination (21). It is possible that this enzyme is also membrane bound and, therefore, has first access to the adenosine that enters the myocardial cell. As illustrated in Figure 1, the intracellular inosine may arise from dephos-
phorylation of inosinic acid or deamination of adenosine moving back into the cell. The concentration and tissue distribution of inosine and inosinic acid in the normal heart are unknown.

As illustrated in Figure 1, adenosine present in the interstitial fluid would dilate the coronary arterioles, and the degree of dilation would be proportional to the adenosine concentration. As previously discussed (11), the adenosine concentration of the interstitial fluid is determined by its rate of release, uptake by the myocardial cell, washout by the perfusing blood, and uptake and inactivation by the red cells (11). During myocardial hypoxia, there is an increased rate of adenosine release (Table 1), a rise in the tissue levels of adenosine (20, 23), a washout of a greater amount of adenosine by the perfusing blood (11) or saline perfusates (24, 25), and a decrease in coronary resistance (10, 11, 23, 25).

The amount of adenosine per gram of myocardium (Table 3) was calculated making two basic assumptions. First, adenosine is found only in the extracellular spaces, and second, the normal pericardial fluid is representative of normal myocardial interstitial fluid. The calculated average value of $2.2 \pm 0.6 (SE) \times 10^{-10}$ moles/g is in close agreement with the value of $2.8 \pm 1.3 (SD) \times 10^{-10}$ moles/g obtained by direct measurement of tissue adenosine content (23). In a previous study (11), a normal adenosine tissue content of the order of $10^{-11}$ moles/g was predicted.

The rate of adenosine release is probably ultimately controlled by the cell concentrations of adenine nucleotides and inorganic phosphate ($P_i$). The rate of hydrolysis of AMP compared to its rate of deamination appears to be controlled by the balance of the concentrations of these substances (17, 18, 26). In-vitro studies with 5'-nucleotidase and AMP-deaminase demonstrate that the quantities of adenosine and inosinic acid produced by these reactions is proportional to the amount of AMP available to these two enzymes which compete for the same substrate. However, ATP exerts an inhibitory effect on 5'-nucleotidase (17, 18) and a stimulatory effect on the AMP-deaminase (26). How the adenosine-generating system may respond to myocardial hypoxia is illustrated in Figure 2 (right side). The AMP concentration increases with myocardial hypoxia, thereby providing more substrate for 5'-nucleotidase whereas the ATP level decreases (27). A decrease in ATP concentration increases 5'-nucleotidase activity and decreases the activity of AMP-deaminase, thereby favoring AMP degradation to adenosine. $P_i$ levels are also increased in myocardial hypoxia (27, 28) providing additional inhibition of AMP-deaminase activity; this will also favor the degradation of AMP to adenosine. With an increase in adenosine formation, the concentration of adenosine in the vicinity of the arterioles should increase and induce vasodilation which in turn would enhance coronary blood flow and the myocardial oxygen supply. The restoration of oxygen balance will accelerate the regeneration of ATP, from AMP, ADP, and $P_i$. Thus, the process is self-regulatory, adenosine functioning as an error signal to indicate the amount of oxygen required by the myocardium.

The coronary blood flow is critically adjusted to a level where the oxygen demands of the myocardium are just met by the supply (2, 3, 5-7). The mechanism that controls myocardial oxygen supply and which may be
mediated by the precise release of adenosine has a parallelism in the mechanism that controls the supply of electrons utilized in oxidative phosphorylation by regulation of acetyl-CoA metabolism (27-35) (left side of Fig. 2). Several of the enzymes of glycolysis, the tricarboxylic acid cycle, fatty acid metabolism, and respiratory electron transport (36) are either inhibited by ATP or stimulated by AMP, ADP, and Pi. Consequently, the low ATP levels associated with high AMP, ADP, and Pi levels that may prevail in myocardial hypoxia will increase the supply of acetyl-CoA to the enzymes of the tricarboxylic acid cycle by augmenting glycolysis (27, 28, 30-35) and possibly by decreasing fatty acid synthesis (32). In addition, the rate of oxidation of acetyl-CoA will be accelerated by activation of tricarboxylic acid cycle enzymes (30, 32) and by an increase in the mitochondrial respiratory rate (36). However, these processes can only proceed if the supply of electrons (left side of Fig. 2) to the respiratory chain is matched by an equivalent supply of oxygen (right side of Fig. 2). In this manner, the regeneration of ATP from AMP, ADP, and Pi would be accelerated and would constitute a self-regulating mechanism.

This scheme could explain the close parallelism between myocardial oxygen balance and coronary blood flow, as observed in reactive hyperemia, arterial hypoxemia, and autoregulation of blood flow. During reactive hyperemia, myocardial oxygen consumption (1), coronary blood flow (1, 11, 23), myocardial release of adenosine (11, 23), and the levels of pyruvate and lactate in the coronary venous blood (1) are increased. The elevated AMP, ADP, and Pi levels and the low ATP levels reached during the preceding ischemic period (27) would stimulate mitochondrial respiration (36) and the release of adenosine (17, 18). Thus, when the circulation is reestablished, the coronary blood flow is increased and oxygen is extracted from the blood at a faster rate. Similarly, during moderate arterial hypoxemia, myocardial oxygen consumption remains constant, and there is an increase in coronary blood flow roughly proportional to the degree of hypoxemia (2, 4, 5). The increased AMP, ADP, and Pi levels and the low ATP, possibly associated with this condition, would increase the mitochondrial respiratory rate (36), thus maintaining a constant rate of extraction of oxygen from extracellular fluid with a lower oxygen concentration (as reflected by the reduced venous oxygen tension) (5). For the same reason, the release of adenosine would be increased, thereby providing the myocardium with a larger flow of blood containing less oxygen. That is, the reciprocal relationship between the effects of AMP, ADP, and Pi with respect to ATP provides a link between myocardial oxygen consumption and coronary blood flow and can account for the parallelism observed between these two variables. In this view, coronary blood flow and oxygen consumption are controlled by a common factor, which is different from the frequently stated view that oxygen consumption is the main determinant of coronary blood flow (2, 6, 8). This hypothesis for the regulation of coronary blood flow does not imply that adenosine is the only determinant of vascular resistance, since there are others known to be involved (9). However, it is suggested that adenosine modulates the effects of all these other determinants and adjusts coronary blood flow to maintain the myocardium in oxygen balance. If, for example, coronary perfusion pressure were elevated so that the myocardium was overperfused, the increased oxygen supply would produce a decrease in the interstitial fluid adenosine concentration and thereby lower coronary blood flow (autoregulation) toward the level where myocardial oxygen balance would be maintained. However, only myocardial hypoxia was known to cause release of adenosine from the heart, and there was no evidence to support the concept that autoregulation of coronary blood flow in response to elevation of coronary perfusion pressure could be due to a reduction in myocardial adenosine concentration. However, the finding of adenosine release from the normal heart makes this aspect of regulation of coronary blood flow feasible, although it
remains to be demonstrated that the basal cardiac adenosine concentration is indeed reduced by excess coronary blood flow.

References
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