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Transmural distribution of extracellular purines in isolated guinea pig heart

(adenosine/endocardium/epicardium/interstitial fluid)

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ABSTRACT **The purine adenosine appears to be involved in regulation of coronary vascular tone. Little is known concerning the levels and distribution of adenosine and related purines in the extracellular fluid of the heart. We have measured epicardial and endocardial levels of adenosine, inosine, hypoxanthine, AMP, and IMP in isolated constant flow perfused guinea pig hearts by using a recently developed technique with porous nylon sampling discs. Venous effluent purine levels were also measured. Concentrations of all purines measured, excluding IMP, were significantly higher in endocardial fluid samples than in epicardial fluid samples ($P < 0.05$). Conversely, IMP levels were significantly lower in endocardial than in epicardial samples. The magnitude of the endocardial/epicardial ratios for adenosine, inosine, hypoxanthine, AMP, and IMP were ≈12:1, 4:1, 5:1, 4:1, and 1:2, respectively. To assess cellular damage, lactate dehydrogenase activity was measured in all fluid samples and was not significantly different in endocardial and epicardial fluid. These data support the existence of significant transmural gradients for extracellular purine levels in crystalloid perfused guinea pig hearts. Transmural differences in vasoactive adenosine levels may be partially due to the greater endocardial oxygen consumption and metabolism and may be involved in maintaining relatively high subendocardial blood flows in the face of high intramyocardial pressures.**

Adenosine may be responsible for modulating myocardial blood flow in response to alterations in oxygen supply and demand (1). This hypothesis predicts a consistent relationship between extracellular adenosine levels and coronary vascular tone or blood flow. In examining this hypothesis, the process of myocardial adenosine formation has been intensively studied (1–4). Unfortunately, most indices of adenosine formation do not directly reflect interstitial adenosine levels—the adenosine pool in direct contact with extracellular receptors. Furthermore, these same indices only reflect global changes in adenosine levels and cannot detect regional differences in extracellular adenosine. The mammalian myocardium is a heterogeneous tissue and the subepicardial and subendocardial regions differ physically, metabolically, and functionally (5–10). Little information is available regarding spatial heterogeneities in extracellular metabolite levels. Interestingly, recent studies indicate that transmural differences exist in the distribution of adenosine-regulating enzymes (11) and it has been demonstrated that transmural gradients exist for intracellular adenosine during control perfusion and ischemia *in vivo* (12). Thus, it is possible that physiological messengers such as adenosine may be unevenly distributed throughout the myocardial interstitial space.

Recently, different methods have been developed for sampling interstitial transudate on the epicardial surface of the myocardium (13–18). In the present study, we measured epicardial and endocardial transudate concentrations of adenosine, inosine, hypoxanthine, AMP, and IMP in the isolated perfused guinea pig heart to determine whether significant transmural gradients do exist for extracellular adenosine and related purines.

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MATERIALS AND METHODS

Isolated Perfused Hearts. Adult male guinea pigs were anesthetized with sodium pentobarbital (50 $mg \cdot kg^{-1}$) administered intraperitoneally. A tracheostomy was performed and positive pressure ventilation was initiated. Following a thoracotomy, the caval veins were ligated, and the hearts were excised and directly placed into ice-cold perfusion fluid prior to aortic cannulation and retrograde perfusion. The hearts were perfused in a nonrecirculating Langendorff mode at a constant flow rate of 5.0 ± 0.1 $ml \cdot min^{-1} \cdot g^{-1}$. Flow was controlled with a peristaltic pump. The perfusate was a modified Krebs–Henseleit bicarbonate buffer containing 119 mM NaCl, 3.2 mM KCl, 1.4 mM $CaCl_2$, 1.20 mM $MgSO_4$, 25.0 mM $NaHCO_3$, 1.2 mM KH_2PO_4, and 11.0 mM glucose equilibrated with 95% O_2/5% CO_2 to yield a pH of 7.40 at 37°C. A cannula was introduced into the right ventricle via the inferior vena cava to allow the collection of coronary venous effluent. The pulmonary artery was ligated to prevent venous leakage onto the ventricular surface. Aortic pressure was continuously monitored via a fluid-filled polyethylene line (i.d., 0.9 mm) attached to a port located ≈5 mm above the aortic cannula. An incision was made in the left atrium and the mitral valves were carefully excised to allow the placement and retrieval of endocardial sampling discs. Hearts were then equilibrated for 45 min prior to experimentation. Oxygen consumption was calculated by collecting perfusate and venous effluent samples in a gas-tight syringe and determining Po_2 with a Corning blood gas analyzer. Myocardial oxygen consumption (MVo_2) was calculated as follows:

$$MVo_2\ (\mu l\ of\ O_2 \cdot min^{-1} \cdot g^{-1}) = (Po_{2p} - Po_{2v}) \times \text{coronary flow} \times (c/760),$$

where Po_{2p} and Po_{2v} refer to coronary perfusate and venous effluent Po_2 values (mmHg; 1 mmHg = 133 Pa), respectively, and $c = 0.0227$ (Bunsen solubility coefficient of oxygen in perfusate at 37°C, ml of $O_2 \cdot atm^{-1} \cdot ml^{-1}$).

The oxygen supply/demand ratio was calculated as

$$Po_{2p}/(Po_{2p} - Po_{2v}).$$

Epicardial and Endocardial Fluid Sampling. Epicardial and endocardial fluid was sampled by using porous nylon discs as described (16–18). Epicardial discs (diameter, 6 mm) were cut from sheets of porous (pore size, 0.45 μm) nylon filters (MicroSep Magna Nylon 66 membrane filters; Micron Separations, Westboro, MA). The discs were preweighed dry

prior to soaking in Krebs–Henseleit solution. Two wetted discs were applied to the left ventricular epicardial surface simultaneously and one disc was placed inside the left ventricle against the endocardial surface of the free ventricular wall. Endocardial and epicardial discs were left in place for 2 min to allow equilibration of disc fluid with epicardial and endocardial fluid. Previous studies (17) indicated that the concentration of adenosine or a nonmetabolizable adenosine analogue in the epicardial discs reached a plateau within 1 min. Adenosine also reached equilibration with the endocardial extracellular fluid in <1 min. After the 2-min contact time, the discs were carefully removed, reweighed, and stored in vials at −80°C until analyzed for purines by HPLC. This process was repeated once for a total collection time of 4 min with four epicardial and two endocardial discs.

HPLC Analysis. Discs were thawed and the sample fluid was eluted with 400 μl of distilled water with the aid of centrifugation. The samples were simultaneously filtered (0.22 μm). A 174-μm sample was then injected onto a C-18 reverse-phase column (Supelco C-18, 5 μm) and eluted using a buffer gradient (100 mM KH_2PO_4/1% methanol, pH 5.53, at time 0 to 100 mM KH_2PO_4/15% methanol, pH 5.58, at 14 min). Elution was complete at 22 min. Absorbance was continuously monitored at 245 nm. The HPLC system consisted of Beckman 110B pumps, a Beckman 166 Programmable Detector, and a Waters 712 WISP autosampler. Peaks were quantitated by comparison of peak areas (determined via integration) with those for standards run routinely with each set of experimental samples. This technique allowed the simultaneous determination of adenosine, inosine, hypoxanthine, IMP, and AMP. The recovery of purine standards applied to discs before analysis was calculated to be 93% ± 4% (n = 40). Venous effluent samples were thawed and 175-μl samples were injected directly onto the column and analyzed in the same manner as described for the epicardial and endocardial samples.

Lactate Dehydrogenase Determination. Levels of lactate dehydrogenase were measured in epicardial, endocardial, and venous fluid samples by monitoring the rate at which pyruvate was reduced to lactate by lactate dehydrogenase. This reaction was coupled to the oxidation of NADH, which was monitored by the change in absorbance at 340 nm (15). Activity is expressed as international units per ml of sample ($IU \cdot ml^{-1}$); 1 unit is equivalent to the amount of enzyme required to degrade 1 μmol of substrate per min at 37°C.

Experimental Protocol. After equilibration, hearts were perfused for an additional 45 min, during which time epicardial, endocardial, and venous effluent samples were obtained every 15 min. Hemodynamic parameters were simultaneously measured. In five hearts, lactate dehydrogenase levels were measured in the disc fluid samples and from the coronary venous effluent at the end of the experimental periods.

Statistical Analysis. Unless stated otherwise, all values shown are means ± SEM. Statistical comparisons between purine values obtained from the three different compartments were made by Student's t test with Bonferroni's correction for multiple comparisons. Significant differences were indicated by $P < 0.05$.

RESULTS

Isolated perfused guinea pig hearts displayed relatively high coronary resistances and low MVo_2 values, indicative of minimal metabolic stress and vasodilation, consistent with the nonisovolumic nature of the preparation (Table 1). Coronary vascular resistance, MVo_2, and the O_2 supply/demand ratio all remained relatively stable throughout the 45-min experimental period (Table 1).

Table 1. Coronary vascular resistance, O_2 supply/demand, and MVo_2 in isolated guinea pig hearts

Time, min	Resistance, $mmHg \cdot ml^{-1} \cdot min^{-1} \cdot g^{-1}$	MVo_2, $\mu l \cdot min^{-1} \cdot g^{-1}$	O_2 supply/ demand
0	9.3 ± 0.6	37.1 ± 2.7	2.2 ± 0.3
15	9.1 ± 0.6	38.4 ± 3.3	2.3 ± 0.1
30	9.0 ± 0.6	41.7 ± 3.1	2.1 ± 0.1
45	8.8 ± 0.6	45.5 ± 3.8	2.0 ± 0.1

Coronary resistance, MVo_2, and O_2 supply/demand values were determined every 15 min during 45 min of constant flow perfusion. All values are means ± SEM (n = 11).

Myocardial extracellular purine levels during 45 min of perfusion are depicted in Fig. 1. All purine concentrations, except IMP, were highest in the endocardial fluid samples and lowest in coronary venous effluent samples. The concentration of IMP was higher in the epicardial fluid than in the

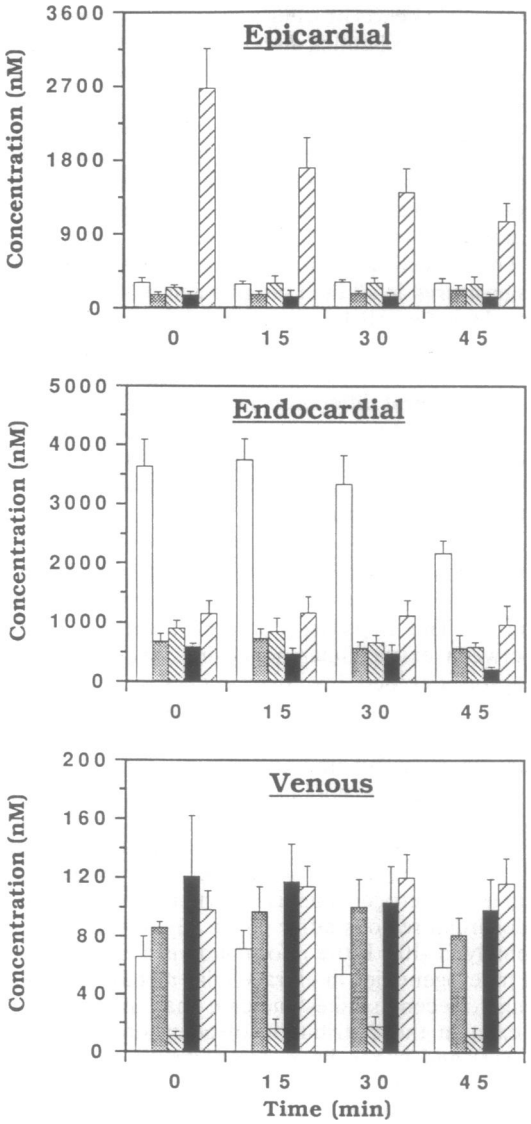

FIG. 1. Epicardial, endocardial, and venous effluent purines during 45 min of constant perfusion. All purine concentrations, excluding IMP, were significantly higher in endocardial samples compared to epicardial samples at all times ($P < 0.05$). In contrast, IMP was significantly higher in epicardial samples compared to endocardial samples ($P < 0.05$). All values shown are means ± SEM (n = 9–11). □, Adenosine; ▨, inosine; ▧, hypoxanthine; ■, AMP; ⧄, IMP.

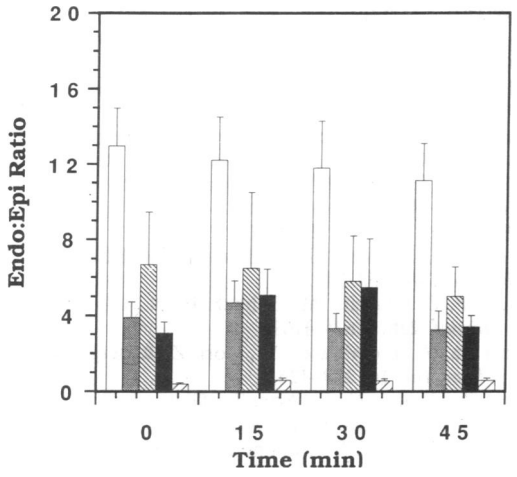

FIG. 2. Endocardial/epicardial ratios for adenosine, inosine, hypoxanthine, AMP, and IMP during 45 min of constant flow perfusion. All values shown are means ± SEM (n = 9–11). □, Adenosine; ▨, inosine; ▧, hypoxanthine; ■, AMP; ▨, IMP.

endocardial fluid during control perfusion. The magnitudes of the endocardial/epicardial ratios for all purines are shown in Fig. 2. The greatest transmural gradient observed was that for adenosine (≈12:1), whereas the gradients for inosine, hypoxanthine, and AMP were much lower (3–5:1). The gradients for all purine compounds remained relatively stable throughout the experimental period (Fig. 2).

Lactate dehydrogenase activities were 296 ± 26, 236 ± 63, and 1 ± 1 IU $\times 10^3$/ml in the epicardium, endocardium, and venous effluents, respectively, during control conditions.

DISCUSSION

These results indicate that significant transmural gradients exist for adenosine and related purine compounds even under control conditions of coronary perfusion in the isolated perfused guinea pig heart (Figs. 1 and 2).

The adenosine hypothesis of metabolic regulation of coronary flow predicts that a causal relationship exists between extracellular adenosine levels and coronary blood flow (1–4). This hypothesis further implies that a consistent correlation exists between the oxygen supply/demand ratio and adenosine formation. Many studies have obtained consistent correlations between coronary flow and adenosine levels and between adenosine formation and various indices of intracellular metabolism (1, 2, 18–20). Unfortunately, almost all of the studies examining these relationships have employed techniques that may not represent accurate indices of interstitial adenosine levels. Furthermore, these indices only reflect global changes in adenosine formation. It has been known for some time that the myocardium is heterogeneous with respect to coronary blood flow and intracellular metabolism (10). Under basal conditions, endocardial blood flow is thought to be slightly higher than or equal to the epicardial blood flow (10, 21). Owing to a variety of factors, including greater wall stresses and intramyocardial pressure (7, 8), and enhanced endocardial metabolism (9, 10, 22, 23), subendocardial oxygen consumption is ≈1.5-fold greater than subepicardial oxygen consumption under normal conditions (5, 6, 10). Since tissue pressure during systole is so much greater in the subendocardium than in the subepicardium, endocardial coronary flow is largely restricted to diastole (7, 8). No differences in endocardial capillary density, distribution, or cross-sectional area exist to account for the increased oxygen requirements in the face of similar absolute flow rates (24). Thus, endocardial tissue P_{O_2} is lower than epicardial P_{O_2}. Since the adenosine hypothesis predicts consistent relationships between extracellular adenosine, coronary flow, and intracellular metabolism (1, 2, 4, 19, 20), these transmural differences may be associated with transmural gradients in extracellular adenosine levels.

These results, obtained with porous discs placed on the epicardial and endocardial surfaces of the left ventricle (16–18), indicated that significant transmural differences do exist in the levels of extracellular adenosine and related purines (Fig. 2). Extracellular purine levels are significantly higher in the endocardial extracellular fluid than in fluid from the epicardial region (Fig. 1), even under conditions of relatively high oxygen delivery relative to demand (O_2 supply/demand > 2.0). These findings are in agreement with documented differences in transmural oxygen supply and demand, coronary flow, and metabolism (7–10). They are also consistent with the recent observation that endocardial levels of free intracellular adenosine are significantly higher than epicardial levels under control conditions in the *in situ* dog heart and that myocardial underperfusion greatly exaggerates this transmural difference (12). Since it is assumed that free intracellular adenosine levels reflect extracellular adenosine levels, the results for intracellular adenosine were thought to be indicative of a gradient for extracellular adenosine (12). The present results support this assumption. The higher endocardial adenosine levels, greater rate of endocardial metabolism, and higher endocardial blood flow under normal conditions are all consistent with the adenosine hypothesis of flow regulation. It remains to be determined whether these consistencies reflect a causal relationship or are simply circumstantial.

Several possibilities might explain the transmural differences of adenosine levels observed in this preparation. These include regional differences in O_2 supply/demand and intracellular metabolism (7–10) and an uneven transmural distribution of the enzymes regulating adenosine formation and/or degradation. A recent examination of the regional distribution of adenosine-regulating enzymes (5′-nucleotidase and adenosine deaminase) in rat, ox, and pig heart demonstrated that only in the ox heart was there a tendency for greater 5′-nucleotidase and lower adenosine deaminase activity in the endocardium relative to the epicardium (11). Even in this species the transmural difference was minimal. In general there were no differences noted in subendocardial versus subepicardial 5′-nucleotidase activities. In all three species examined, the ratio of 5′-nucleotidase/adenosine deaminase was significantly greater in the epicardial layers than in the mid-myocardium (11). Thus, the processes responsible for net adenosine formation are greatest in the epicardial and endocardial layers and lowest in the mid-myocardium. The ratio was approximately equal in the subendocardial and subepicardial regions. While such an enzyme distribution does not explain the results observed in the present study, it remains possible that species differences exist in transmural distribution of these enzymes. This might not be surprising based on the significantly different myocardial activities of these enzymes in different species (25) and the different characteristics of purine transport in guinea pig and rat hearts.

It is interesting to note that the transmural gradient for IMP was reversed in relation to the gradients for all other metabolites (endocardial/epicardial gradient, 0.5). This is entirely consistent with the recent observation that AMP deaminase activity is almost 2-fold greater in the subepicardium than in the subendocardium of the rat heart (11). The fact that large levels of IMP are present in the extracellular fluid indicates that the previous measurement of venous inosine and hypoxanthine as metabolic products of adenosine does not necessarily result in an accurate estimate of total adenosine formation. These large levels of IMP would conceivably

contribute to the extracellular levels of inosine and hypoxanthine in these hearts.

It is possible that the transmural differences observed are a result of artificial tissue damage or disruption due to the placement of the sampling discs. However, if this were so, the gradients for the various purines would likely be similar in magnitude. This is not the case since IMP is actually lower in the endocardial extracellular fluid than in the epicardial fluid, and the gradients for the other purines are much lower than the gradient for adenosine. Furthermore, lactate dehydrogenase activity was similar in both the epicardial and endocardial fluid samples. There are no known transmural differences in the distribution of lactate dehydrogenase in other species (26). Hence, these results indicate that cellular disruption (if any) must be comparable in both compartments. The higher concentrations of lactate dehydrogenase in the epicardial and endocardial fluid samples relative to the venous effluent indicate that epicardial and endocardial fluid samples represent much better indices of interstitial solute concentrations than do venous samples, as recently discussed by Weinen and Kammermeier (27).

In an isolated heart perfused at constant flow with a crystalloid solution, it is possible that the high endocardial purine levels are caused by an inadequate oxygen supply to the endocardium. However, as much as a 30% increase in flow (data not shown) did not change the purine values for epicardium and endocardium. Moreover, adenosine concentrations of 0.3 μM in the epicardium and of 3.6 μM in the endocardium are consistent with an average left ventricular transmural adenosine concentration of 1.0 μM found in the *in situ* blood perfused dog heart by the microdialysis technique (28). Nevertheless, an arterial adenosine concentration of 1 μM has been reported to produce maximal vasodilation (29). This observation may not be applicable to endogenous adenosine because (*i*) the response to intraarterial adenosine is in part due to an endothelial-mediated factor (30), (*ii*) there are different diffusion distances from source to vascular smooth muscle for intravascular and interstitial adenosine, (*iii*) there are possible differences in adenosine sensitivity between the intraluminal and the extraluminal surfaces of the coronary vascular smooth muscle, and (*iv*) arterial adenosine concentrations as high as 12 μM only result in a 0.4 μM concentration in the epicardial fluid (17).

In conclusion, this study indicates that significant transmural gradients exist for adenosine, inosine, hypoxanthine, AMP, and IMP during control conditions in isolated perfused guinea pig hearts. While the physiological significance of these regional differences in extracellular purine levels is not known, it is possible that enhanced endocardial adenosine levels aid in maintaining adequate endocardial perfusion in the face of greater degrees of endocardial metabolism and extravascular compression.

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