Adenosine Release by the Isolated Guinea Pig Heart in Response to Isoproterenol, Acetylcholine, and Acidosis: The Minimal Role of Vascular Endothelium

Hubert Bardenheuer, Bridget Whelton, and Harvey V. Sparks Jr.

The objective of this study was to determine the contribution of endothelial cells to adenosine appearing in venous effluent of isolated perfused guinea pig hearts. The adenine nucleotide pool of endothelial cells was selectively labelled by infusing $^3$H-adenosine ($5 \times 10^{-7}$ M) into the heart for 30 minutes. Selective labelling of the endothelial adenine nucleotides was confirmed by measuring the relative specific activities of the nucleotides of coronary endothelial cells (removed from the heart by enzyme treatment). Endothelial ATP, ADP, and AMP had relative specific activities that were 49, 25, and 7 times higher, respectively, than their nucleotide counterparts in total myocardial tissue. Isoproterenol increased the release of both total adenosine and radioactive adenosine, but the relative specific activity of venous adenosine decreased dramatically. Acetylcholine, at a concentration that caused no change in left ventricular pressure but caused a decrease in coronary vascular resistance, increased the release of total adenosine. However, both radioactive adenosine release and the relative specific activity of venous effluent adenosine were decreased with acetylcholine. Infusion of hydrochloric acid caused a sustained reduction in left ventricular pressure and coronary vascular resistance. Total adenosine release fell within one minute and remained reduced during HCl. Radioactive adenosine release was elevated at 15 seconds but fell below control values at 2 minutes and remained reduced during steady-state acidosis. We conclude that the majority of the adenosine released in response to isoproterenol and acetylcholine originates from an unlabelled compartment, most likely the myocytes. Acidosis results in decreased release of adenosine from both the labelled endothelium and the unlabelled cells in the heart. We conclude that the coronary endothelium contributes minimally to the release of adenosine into coronary venous effluent under the conditions of our experiments.

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addition, we tested the hypothesis that coronary endothelium releases adenosine in response to acetylcholine (ACH), an agent that has been shown to increase coronary effluent adenosine that is suspected to be of endothelial origin.  

**Materials and Methods**

**Isolated Heart Preparation**

Male guinea pigs (300–400 g) were stunned by a blow to the head, and their hearts were removed and perfused using a modified Langendorf preparation, i.e., retrograde perfusion via the aorta with a noncirculating physiologic salt solution (PSS). The composition of the PSS was as follows (mM): NaCl 127, KCl 4.7, MgSO₄ 1.1, CaCl₂ 2.5, NaHCO₃ 25, glucose 5.5, Na-Pyruvate 2.0.  

The perfusate was equilibrated with 95% O₂–5% CO₂ at 37°C, pH 7.40. A latex balloon was placed in the left ventricle, and the following parameters were measured and recorded on a Grass polygraph (Grass Instruments, Quincy, Mass.): 1) left ventricular pressure (LVP), measured with a Statham P23 ID pressure transducer (Statham Instruments, Los Angeles); 2) heart rate, triggered from LVP; 3) coronary perfusion pressure, measured with a Statham P23 AC pressure transducer located at the level of the aortic cannula; and 4) coronary flow, measured by means of an electromagnetic flow probe (Zepeida, Seattle, Wash.) placed in the aortic cannula. The hearts were electrically paced at 280 beats/min with a Grass S9 stimulator. Coronary vascular resistance (CVR) was calculated as perfusion pressure divided by the flow rate. After an equilibration period of about 20 minutes, during which time the hearts were perfused at constant pressure (46 mm Hg), perfusion was switched to constant flow at 10 ml/min. Tritiated adenosine (³H-adenosine) was infused for 30 minutes at a delivered concentration of 5 x 10⁻⁴ M and specific activity of 1,351 pCi/pmol. Following the ³H-adenosine infusion, constant-flow perfusion was continued (ACH and HCl experiments), or perfusion was switched to constant pressure (ISO), and 30 minutes were allowed for washout of tracer adenosine from the extracellular compartment. The washout period was followed by one of our experimental maneuvers.

**Total Myocardial and Endothelial Cell Relative Specific Activity Measurements**

To confirm that the labelling procedure results in selective labelling of the endothelial cells, we compared the relative specific activities of the adenine nucleotides (ATP, ADP, and AMP) in the endothelial cells with those in total myocardial tissue. After the labelling and washout periods, the endothelial cells were removed using a modified technique of Nees et al.  

Prelabelled hearts were immersed in a graduated cylinder containing 20 ml DMEM (Dulbecco’s Modified Eagle Medium; Gibco, Long Island, N.Y.) with 20% sucrose at 37°C. Following immersion, flow through the heart was reduced to 1 ml/min, and perfusion was switched to PSS containing 0.1% collagenase and trypsin. After 15 minutes, the perfusion medium was switched back to PSS without enzyme, and flow was then intermittently increased and decreased to aid in dislodging the loosened endothelium. A total volume of 60 ml was collected in the cylinder. To decrease metabolism, all steps after detachment of the cells were performed at 4°C. The crude endothelial harvest was centrifuged at 250 g for 10 minutes, and the sedimented cells were resuspended with DMEM and centrifuged again. This was repeated twice. Washed endothelial cells from 3 to 7 hearts were pooled, resuspended in 2 ml DMEM, and transferred to the top of a preformed Percoll density gradient. After centrifugation at 100g for 30 minutes, the endothelial layer was aspirated from the top of the density gradient and added to a separate test tube, along with 15 ml DMEM. The cells were centrifuged for the last time at 250g for 10 minutes. Thereafter, 20 µl 0.5N HCl was added to the endothelial cell pellet, and the deproteinated cells were stored at −20°C until the adenine nucleotides were measured by high-performance liquid chromatography (HPLC) (see below).

To verify that the enzymatic perfusion technique described above selectively isolates endothelial cells, the technique was performed in a separate series of experiments on hearts that were not prelabelled. Instead of deproteinating the final cell pellet, the cells were resuspended in DMEM, plated onto Petri dishes, and incubated in a standard tissue culture environment (DMEM supplemented with l-glutamine and 15% fetal calf serum at 37°C). The cells grew to confluence within 1 week and had the typical unique endothelial morphology: they demonstrated contact inhibition, formed monolayers, and exhibited a cobblestone appearance.

To determine their effect on the heart, after the labelling and washout periods, one of the following drugs was infused at 50 µl/min for 8.5 minutes into the aortic cannula to achieve the delivered concentrations listed: ISO at 5 x 10⁻⁴ M, ACh at 5 x 10⁻⁷ M, HCl at 10⁻³ N (arterial pH 6.8, Paco, 60 mm Hg), or normal saline solution as a vehicle control. The ISO experiments were performed at constant pressure and the hearts were not paced. The ACh and HCl experiments were performed with constant flow (10 ml/min), and the hearts were electrically paced at 280 beats/min with a Grass S9 stimulator. Separate vehicle control exper-
ments were performed using the protocol for the ISO experiments and that of the ACh and HCl experiments. Coronary venous effluent samples were taken immediately prior to drug infusion, during, and 10 (ISO) or 15 (ACh and HCl) minutes after the infusion was stopped. Saline infusion did not alter any of the parameters measured (i.e., LVP, resistance, release of total adenosine, and release of radioactive adenosine), and the results are not shown.

**Measurement of Purine Nucleotides and Nucleosides**

The nucleotides of both total heart tissue and endothelial cells were quantitated by HPLC. The pumps were programmed for gradient elution of 200-μl samples, which were automatically injected onto an NH4 column (Alltech, Deerfield, Ill., ODS 5, i.d. 4 x 250 mm). The linear gradient started with 97% 2.5 mM NH4PO4, pH 3.0, and 3% 750 mM NH4PO4 buffer, pH 3.8. At a flow rate of 1.7 ml/min, the eluate increased to 100% of 750 mM NH4PO4 over 30 minutes. This was followed by a reversal of the gradient to initial conditions over the next 5 minutes. Absorbance of the column eluate was continuously monitored at 254 nm and recorded. ATP, ADP, and AMP were identified by identical correspondence with the retention times of the respective standards. Because of the difficulty in quantitating AMP (many substances coelute, making peak identification difficult), the effluent associated with the AMP peak was collected, and 5′-nucleotidase (EC: 3.1.3.5) was added to degrade the nucleotide to adenosine. The peak-shifted samples were rechromatographed on a C-18 HPLC column and the adenosine quantitated (see below). In addition, the HPLC effluent corresponding to ATP, ADP, and AMP was collected separately for determination of the radioactivity associated with each nucleotide. The specific activity of each compound was calculated by dividing radioactivity in the mass (cpm/pmol). Relative specific activity was calculated by dividing the specific activity of each measured nucleotide by the specific activity of the 3H-adenosine that was infused to label the heart.

Total adenosine release was determined by HPLC, using a C-18 reverse-phase column (Alltech, ultrasphere ODS 4M i.d. 4.6 x 250 mm). The system was programmed for a linear gradient elution starting with 100% 4 mM KH2PO4, pH 4.6, and increasing to 39% 70/30 methanol (vol/vol) in 20 minutes at a flow rate of 1.1 ml/min. Adenosine was identified by comparison of its retention time with an adenosine standard. Nucleoside identification was verified by enzymatically shifting adenosine to inosine with adenosine deaminase (EC: 3.5.4.4) and then rechromatographing the peak-shifted compound. Recovery of added adenosine was greater than 90%. Fractions of the eluate corresponding to the optically determined peaks were collected to determine the radioactivity associated with adenosine, and both specific activities and relative specific activities were calculated.

**Chemicals**

2,8-3H-Adenosine, 40 mCi/mmol, was purchased from ICN, Chemical and Radioisotope Division, Irvine, Calif.; Isuprel hydrochloride was purchased from Breon Laboratories, Sterling Drug Company, New York; all other chemicals were obtained from Sigma Chemical Company, St. Louis, Mo.

**Statistics**

Results are expressed as mean ± SEM. Data were analyzed using the Student's t test to compare the nucleotides in endothelial cells and total myocardial tissue (p values are given in "Results"), and a two-way analysis of variance and Duncan's test for significance were used. Statistical significance is at the p<0.05 level.

**Results**

The specific activities of adenine nucleotides in endothelial cells and in total myocardial tissue are shown in Table 1. We found the specific activities of ATP, ADP, and AMP to be 49, 25, and 7 times higher, respectively, in the endothelial cells than in total myocardial tissue (the latter including the intact endothelium). In the endothelium, the AMP relative specific activity was significantly greater than that of ADP and ATP. In total myocardial tissue, the pattern of specific activities was AMP > ADP > ATP.

ISO infusion at 5 x 10^{-9} M caused a significant increase in LVP and a decrease in CVR as shown in Table 2. The top panel in Figure 1 shows that with ISO, total adenosine release increased and followed the phasic pattern described by DeWitt et al.13 The release of 3H-adenosine increased from 694 ± 148 in control to 1,337 ± 324 dpm/min/g at 2 minutes, or a factor of 1.85. At the same time, total adenosine release increased by a factor of 14.2 (from 52.8 ± 12.1 to 754.2 ± 120.8 pmol/min/g). The result of the dis-

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<th>Table 1. Relative Specific Activities of ATP, ADP, and AMP in Coronary Endothelial Cells and Total Myocardial Tissues</th>
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<tr>
<td>Endothelial cells</td>
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<td>Total myocardial tissue</td>
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Results are expressed as mean ± SEM; n = 6-11. *Significantly different from the corresponding nucleotide in total myocardial tissue; †significantly different from ATP.
Table 2. Hemodynamic Changes in Response to Isoproterenol, Acetylcholine, and Hydrochloric Acid

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<th>Left ventricular pressure (mm Hg)</th>
<th>Coronary vascular resistance (mm Hg/ml/min)</th>
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<tr>
<td></td>
<td>Control</td>
<td>15</td>
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<tr>
<td>ISO</td>
<td>77.7±5.0</td>
<td>81.5</td>
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<tr>
<td>ACh</td>
<td>115.2±4.1</td>
<td>109.5</td>
</tr>
<tr>
<td>HCl</td>
<td>91.7±10.5</td>
<td>35.5*</td>
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<tr>
<td>Control</td>
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<td>7.23</td>
</tr>
<tr>
<td>ACh</td>
<td>6.79±0.36</td>
<td>5.40*</td>
</tr>
<tr>
<td>HCl</td>
<td>6.05±0.57</td>
<td>5.66</td>
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Control parameters were measured immediately prior to drug infusion and postcontrol parameters were measured 10 minutes (ISO, isoproterenol) or 15 minutes (ACh, acetylcholine; HCl, hydrochloric acid) after the infusion was stopped. Results are expressed as mean ± SEM. *Significantly different from control at p<0.05 level; n = 6 for ISO, n = 5 for ACh and HCl.

A proportionate increase in the release of total and radioactive adenosine was a decrease in the specific activity as shown in the lower panel of Figure 1. When the infusion was stopped, all parameters returned to their pre-ISO values.

The results of experiments with ACh infusion are summarized in Figure 2 and Table 2. LVP was not altered in the paced hearts with $5 \times 10^{-7}$ M ACh, but CVR decreased during steady-state infusion. We found an increase in total adenosine release from the heart (Figure 2, top panel) and a decrease in $^3$H-adenosine from 497±133 in control to 223±35 dpm/min/g after 8 minutes ($p<0.05$). The result of increased total adenosine and decreased $^3$H-adenosine release was a fall in specific activity.

The effect of acidosis on the heart is shown in Table 2 and Figure 3. CVR decreased after 30 seconds and remained reduced during infusion but recovered after HCl withdrawal. LVP development fell dramatically within 15 seconds and remained depressed throughout acidosis but recovered when the infusion was stopped. Along with the decline in cardiac function, total adenosine release decreased but returned to control 15 minutes after HCl. Tritiated adenosine release increased significantly from 415±86 in control to 656±71 dpm/min/g at 15 seconds of HCl but fell below control to 233±17 at 2 minutes and did not return to the control value. The specific activity of venous adenosine remained unchanged until 8 minutes of

![Figure 1](http://circres.ahajournals.org/)

**Figure 1. The effect of isoproterenol on total adenosine release (top panel) and relative specific activity of venous adenosine (lower panel). ISO infusion into coronary vasculature began at time 0 and continued for 8.5 minutes. Control samples were collected immediately prior to drug infusion, and postcontrol samples were collected 10 minutes after the infusion was stopped. Results expressed as mean ± SEM. *Significantly different from control at p<0.05; n = 6.**
FIGURE 2. The effect of acetylcholine on total adenosine release (top panel) and the relative specific activity of venous adenosine (lower panel). ACh infusion began at time 0 and continued for 8.5 minutes. Control samples were taken immediately prior to ACh infusion, and postcontrol samples were taken 15 minutes after the infusion was stopped. Results expressed as mean ± SEM. *Significantly different from control at p<0.05, n = 5.

FIGURE 3. The effect of acidosis on total adenosine release (top panel) and tritiated adenosine release (lower panel). HCl infusion began at time 0 and continued for 8.5 minutes. Control samples were collected immediately prior to HCl infusion and postcontrol samples were taken 15 minutes post-HCl. Results expressed as mean ± SEM. *Significantly different from control at p<0.05, n = 5.

Discussion
The purpose of this study was to determine if endothelial cells are responsible for release of adenosine by the heart under specific conditions. Because the nucleoside affects many functions in the heart, knowledge of adenosine regulation, including its cellular origin, is critical to understanding how several important processes in the heart are controlled.

It is well-established that adenosine is formed and released from the heart under different metabolic conditions. However, the cellular site of production of the nucleoside has been unknown. Schrader et al demonstrated in 1976 that more than one compartment is involved in adenosine production. They labelled isolated hearts with radioactive nucleotide precursors and found that hypoxia caused a dramatic increase in the release of unlabelled adenosine and a fall in relative specific activity of adenosine in the venous effluent.

Acidosis when it fell from a control value of 9.38 ± 3.02 dpm/min/g to 5.54 ± 1.66 and did not return to control 30 minutes after switching back to normal PSS.

They concluded that limited oxygen delivery stimulates the formation of the nucleoside from an unlabelled precursor pool. At the time, the nature of the compartmentation was unclear, i.e., whether it was cellular or subcellular. Recently, Nees et al established that the labelling protocol used by Schrader's group results in cellular compartmentation of the radioactivity. They infused radiolabelled adenosine in isolated perfused guinea pig heart and then enzymatically removed approximately 8% of the endothelial cells and calculated that 100% of the label was in the endothelium, incorporated into the adenine nucleotides (90%) and other purine compounds. In addition, Nees' group has demonstrated by autoradiography that infusion of the same concentration of radioactive adenosine results in 95% of the label being sequestered by the endothelium. It appears that in Schrader's early work, the compartments that were being monitored were the labelled endothelial cells and the rest of the unlabelled cells in the heart. Because the myocytes contain 95% of the total myocardial adenine nucleotides, it is likely that the unlabelled adenosine comes from the myocytes. Reinterpretation of Schrader's early work, based on current understanding of the cellular distribution of the labelled purine compounds, is that both the endothelium and myocytes release adenosine under specific conditions.
control conditions and that hypoxia greatly enhances the release of adenosine from myocytes.\textsuperscript{21} In our study, we verified that infusion of a low concentration of radioactive adenosine into isolated hearts results in preferential labelling of the endothelium. Our finding that the specific activities of the endothelial cell nucleotides are much greater than those of total myocardial tissue is consistent with preferential labelling of endothelial cells. In contrast to cultured endothelial cells in which labelling results in equal specific activities of the adenine nucleotides,\textsuperscript{10} we found that ATP, ADP, and AMP are not equally labelled. In the endothelial cells, ATP and ADP had equivalent specific activities, but the AMP pool was labelled to a greater degree. Because of the extreme conditions under which the endothelium was removed from the heart, the specific activities of the final cell pellet may not accurately reflect the situation in situ. However, in total myocardial tissue (which should accurately reflect the labelling patterns of the nucleotides), all three nucleotides had different specific activities with AMP > ADP > ATP. One explanation for the unequal label distribution is that because of the different pool sizes (ATP > ADP > AMP),\textsuperscript{10} 1 hour of labelling and washout is insufficient for complete label equilibration in the in situ endothelium. Another possibility is that there may be pools of ATP and ADP that are relatively inaccessible to the label compared with AMP. Distinct nucleotide pools exist in platelets, with ATP and ADP being sequestered by α-granules.\textsuperscript{22} However, there is no evidence for an equivalent structure in the endothelium. Actin filaments bind ADP in smooth muscle,\textsuperscript{23} and endothelial cells contain actin, so it is possible that this could be the slowly equilibrating ADP pool. However, there is no evidence for intracellular compartmentation of ATP in the endothelium. Regardless of the reason for unequal labelling of the nucleotides, our data substantiate the conclusion of Nees and Gerlach\textsuperscript{10} that it is possible to achieve preferential labelling of endothelial cells.

In this study, we used changes in the specific activity of adenosine in the venous effluent to estimate changes in the relative contribution of endothelial cells and unlabelled compartments. Compared with the specific activity of venous adenosine during control, if the specific activity increased in response to an agonist, the conclusion is that the labelled endothelium has increased contribution to the adenosine being release. If the specific activity decreased with an agonist, the conclusion is that there is increased contribution by an unlabelled compartment to the nucleoside release. Cardiac myocytes are by far the most prominent cell type in the heart and are probably the source of most unlabelled adenosine.

The effects of β-adrenergic agonists on the release of adenosine by the isolated perfused heart are well documented.\textsuperscript{13, 18} The increase in LVP and fall in CVR are accompanied by elevated adenosine release that is phasic in nature. Because the augmented work by the heart causes increased ATP use and could result in elevated adenosine production,\textsuperscript{14} it seems likely that the myocytes are the origin of the nucleoside released in response to catecholamines. However, the endothelium is another possible source of adenosine because coronary endothelial cells in vitro release adenosine in response to β-adrenergic agonists.\textsuperscript{10} Based on our study, we reject the hypothesis that a quantitatively significant proportion of venous adenosine released in response to ISO originates from the endothelium. We found instead that ISO stimulates the release of the nucleoside from an unlabelled compartment to a much greater degree than it stimulates adenosine release from the labelled endothelium.

ACh causes coronary vasodilation and its ability to relax large coronary arteries is mediated by endothelial cells.\textsuperscript{25} Schrader et al\textsuperscript{12} demonstrated that ACh causes the release of adenine nucleotides in isolated guinea pig hearts, and they were able to inhibit the ACh-induced dilation by 40% with the adenosine receptor antagonist theophylline. Based on these data, the latter group suggested that ACh stimulates the endothelium to release nucleotides that can be degraded to adenosine by ecto-phosphatases. The adenosine, in turn, could act on the smooth muscle and cause relaxation. If this hypothesis is correct, there should be an increase in radioactive adenosine release from the heart in response to ACh. In our study, the concentration of ACh used caused no change in LVP development but did cause decreased CVR and enhanced adenosine release. Since radioactive adenosine release decreased, we conclude that ACh does not stimulate endothelial adenosine release, nor is there increased extracellular formation of adenosine from endothelial nucleotides. Instead, it appears that the nucleotides released from the heart with ACh come from the unlabelled myocytes. In contrast to our results, in a recent study using a similar endothelial prelabelling technique, Deussen et al\textsuperscript{27} found that ACh caused an increase in radioactive adenosine release when flow was allowed to increase, i.e., with constant pressure perfusion. A possible explanation for the difference in our results with respect to the release of radioactive adenosine is that the increased flow in experiments of Deussen et al caused the washout of radioactivity. This possibility is supported by the fact that although the release of radioactive adenosine increased, the concentration of radioactive adenosine fell. On the other hand, their control experiments with other vasodilators suggested that the release was specific for ACh. This raises the possibility that endothelial-cell adenosine release occurs in response to ACh only when flow is increased. However, despite the differences in the method of perfusion between our study and Deussen’s, both studies showed an increase in the release of total adenosine from the heart and a decrease in the relative specific activity of venous adenosine. Therefore, both studies indicate that during ACh stimulation, the adenosine appearing in the venous effluent originates primarily from the myocytes.

We found acidosis to cause a dramatic decrease in LVP development and a fall in vascular resistance. Accompanying the fall in cardiac function was a decrease in total adenosine release from the heart. In
In contrast, Mustafa\textsuperscript{22} reported an increase in adenosine release with decreased pH in the isolated rabbit heart. The two studies differ in the species studied, in the means of achieving acidosis (both H\textsuperscript{+} and Paco\textsubscript{2} were altered in our study, while Mustafa elevated Paco\textsubscript{2} with H\textsuperscript{+} constant and vice versa), and in the manner in which the isolated hearts were perfused (our experiments were performed at constant flow, while theirs were free flow). Deussen et al\textsuperscript{21} found that total adenosine release increased slightly with a less acidic insult than in our study, i.e., arterial pH 7.18 and Paco\textsubscript{2} 58 mm Hg, and with constant pressure perfusion.

Acidosis causes a sustained increase in adenosine release from cultured coronary endothelial cells.\textsuperscript{10} We did not find a sustained release of adenosine from in situ endothelium but found instead an early, transient increase in radioactive adenosine that was followed by a decrease below control in the steady state. Deussen et al\textsuperscript{21} found no increase in radioactive adenosine release from the heart. The group found a decrease in the relative specific activity of venous adenosine, so both Deussen’s study and ours indicate that the majority of the adenosine being released during acidosis originates from the myocyte. We do not know why endothelial cells in the heart do not respond in the same fashion as cultured cells; perhaps the presence of the cardiomyocytes alters endothelial function. We found that 15 minutes post-HCl-infusion endothelial adenosine release was still depressed, but both LVP and the release of adenosine from the unlabelled compartment recovered. This implies that the impairment of endothelial function by acidosis is more long lived than is the impairment of myocyte function.

In summary, we have 1) confirmed that infusion of a low concentration of radioactive adenosine results in selective labelling of the vascular endothelium and 2) used this technique to assess the contribution of endothelium to altered adenosine release from the heart. We conclude that in the presence of ISO and ACh, both of which increase adenosine release, the majority of the nucleoside originates from the myocytes. During acidosis, adenosine release diminishes as a result of decreases from both the myocytes and the endothelial cells. These studies point to a minimal role for the endothelium in augmenting venous adenosine release in response to ISO, ACh, and acidosis.

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