Transendothelial Transport and Metabolism of Adenosine and Inosine in the Intact Rat Aorta

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This study was aimed at defining the role of vascular endothelium in the transport and metabolism of adenosine. For this purpose, endothelium-intact and endothelium-denuded isolated rat aortas, perfused at constant flow (2 ml/min), were prelabeled with 3H-adenosine or 3H-inosine for 10 minutes at concentrations of 0.012-100 μM. Sequestration of adenosine by endothelium was determined from radioactivity recovered during selective endothelial cell removal with deoxycholic acid (0.75% for 15 seconds). In the physiological concentration range of adenosine (0.012-1 μM), fractional sequestration by endothelium was 90-92% of the total adenosine incorporation by the aorta. Endothelial sequestration of inosine at 0.1 μM was 85%. At 100 μM adenosine or inosine, fractional sequestration by aortic endothelium was 33% and 39%, respectively. Analysis of the specific radioactivity of adenine nucleotides extracted from prelabeled aortas indicated that most of the adenosine was incorporated into endothelial adenine nucleotides. Incorporation of inosine into endothelial ATP was approximately 15% that of adenosine. Inhibition of aortic adenosine deaminase with erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) did not influence sequestration of 0.1 μM adenosine, but resulted in a 49% reduction of total endothelial incorporation at 100 μM adenosine. Transfer of radioactive purines from the endothelium to underlying smooth muscle after prelabeling was equivalent to only 1%/hr of total endothelial radioactivity. Our findings suggested that 1) macrovascular endothelium of the aorta constitutes a highly effective metabolic barrier for circulating adenosine and inosine; 2) transfer of labeled adenine nucleotides from endothelium to underlying smooth muscle is rather small and most likely proceeds via dephosphorylated purine compounds; and 3) measurement of adenosine trapping in endothelial and smooth muscle compartments overestimates the transendothelial adenosine concentration gradient. (Circulation Research 1989;64:1147-1157)

Adenosine is an important metabolite in the catabolism of adenine nucleotides and the hydrolysis of 3'-adenosylhomocysteine. It is a potent coronary vasodilator,1 and its other known actions in the heart include antagonism of β-adrenergic-mediated inotropic stimulation,2 inhibition of electrical conduction in the atrioventricular node,3 and stimulation of coronary endothelial adenylate cyclase.4 The heart itself may produce adenosine in sufficient quantities to bring about these same responses. While cardiomyocytes have recently been reported to produce the majority (86%) of adenosine released by the heart under normal conditions,4 coronary endothelial cells are also known to play a crucial role in regulation of the extracellular concentration of adenosine due to rapid uptake and metabolism. At physiological concentrations (0.05-1.0 μM), extraction of adenosine from the vascular space during single passage through the heart is approximately 70%, and it appears that 80-90% of adenosine taken up by the heart is rapidly phosphorylated and retained within the coronary endothelium in the form of adenine nucleotides.5 Thus, coronary endothelium constitutes an effective metabolic barrier for the transport of adenosine between the vascular and interstitial spaces of the myocardium. Endothelial trapping of radiolabeled adenosine led Nees and coworkers3 to propose that the vasodilator action of infused adenosine is mediated by the coronary endothelium since adenosine was thought not to reach vascular smooth muscle. As an alternative explanation, Sparks and coworkers6 pro-
posed that the endothelial barrier for adenosine is not complete and that vascular smooth muscle sensitivity to adenosine is higher than adenosine infusion studies have indicated. However, no direct measurements are available of the adenosine concentration at smooth muscle cell receptors when the vascular concentration of this nucleoside is altered. The magnitude of the endothelial concentration gradient for adenosine depends not only on the permeability of the endothelial layer for adenosine, but also on the kinetic parameters for adenosine uptake from the subendothelial space by endothelial and smooth muscle cells. Moreover, all previous studies have been carried out on capillary endothelium, and it is not known if endothelium overlying vascular smooth muscle also represents a barrier for adenosine transport or if both vascular cells function as a single compartment in the metabolism of adenosine.

A possible mechanism proposed to explain the transfer of the adenosine vasodilator signal from vessel lumen across the endothelium to smooth muscle is that these two cellular compartments are metabolically coupled via gap junctions, possibly involving cyclic AMP. This hypothesis was suggested by the parallel activation of coronary endothelial adenylate cyclase and increased coronary flow caused by adenosine and its derivatives 5'-N-ethyl-carboximide-adenosine (NECA) and (−)-N²-(R-phenyl-isopropyl)-adenosine (PLA) in the guinea pig heart. In support of this hypothesis is the recent observation of gap junctional transfer of dye and radioactive uridine derivatives from endothelium to vascular smooth muscle cells in coculture experiments.

For investigation of transport and metabolism of adenosine and inosine in endothelial and vascular smooth muscle cells in their normal in vivo relation, the isolated, perfused rat aorta was used as a convenient experimental model. The results obtained permitted an estimate of the magnitude of the adenosine concentration gradient across the intact endothelium. Furthermore, this experimental model made possible the direct study of metabolic coupling between endothelium and smooth muscle by chemical measurement of the transfer of endothelial adenine nucleotides to underlying smooth muscle.

Materials and Methods

Aorta Preparation

A total of 99 Wistar rats of either sex, weighing 300–350 g, were anesthetized with ether and killed by a blow to the head. The chest was opened, and the descending thoracic aorta was cannulated and perfused at a constant flow rate of 2 ml/min with a medium equilibrated with room air at a temperature of 37° C and containing (mM) NaCl 140, KCl 2.7, CaCl₂ 1.8, MgCl₂ 1.03, NaH₂PO₄ 0.42, glucose 5.0, pyruvate 2.0, and HEPES 10.0 and bovine albumin 2 g/l (pH 7.40). The abdominal aorta was cannulated at the level of the renal arteries. The cannulas were mounted on a rigid holder, and the approximately 3.5-cm-long aortic segment was excised and placed into a saline (0.9%) bath at 37° C. Fat and connective tissue were dissected away, and the intercostal arteries were ligated with fine silk sutures. The outlet tubing height was 40 cm H₂O. The vessels were elevated briefly above the surface of the saline bath, and only preparations judged visually to be free of leaks were used in experiments. For experiments involving radioactive prelabeling with ³H-adenosine (see below), a cylindrical chamber (volume 2.7 ml) was sealed around the vessel and saline was continuously withdrawn past the outside of the aorta at a rate of 1 ml/min.

³H-Adenosine Prelabeling

For prelabeling of the adenine nucleotides of the vascular endothelium, the perfusion medium was changed for 10 minutes to one containing 0.5 µCi/ml [2,8,5'-³H]adenosine (³H-adenosine) (specific radioactivity 40 Ci/mmol) (Amersham, Arlington Heights, Illinois), yielding an adenosine concentration of 12 nM. Unlabeled adenosine was added to the prelabeling medium to achieve concentrations of 100 nM–100 µM. During prelabeling and the subsequent 15-minute washout period, saline withdrawn through the chamber sealed around the aorta was collected and counted for radioactivity. Total radioactivity escaping from the adventitial side of the aortic wall averaged less than 0.08% of the radioactivity used to prelabel these vessels. In additional experiments, [2,8,5'-³H]inosine (³H-inosine) (prepared from ³H-adenosine by use of adenosine deaminase) was used for prelabeling at concentrations of 0.012, 0.10, 1.0, 10, and 100 µM as described above for ³H-adenosine prelabeling.

Aortas were prelabeled either with an intact endothelium 10–15 minutes after completion of the preparation or 30 minutes after removal of the endothelial cell layer. For selective removal of the vascular endothelium, aortas were perfused for 15 seconds with a solution of 0.75% deoxycholic acid (DCA) in distilled water at a flow rate of 6 ml/min, followed by perfusion with the normal medium at a rate of 6 ml/min for 3 minutes. In preparations that were previously prelabeled with ³H-adenosine, perfusion was stopped after 3 minutes and the aorta was rapidly removed from the cannulas, frozen in liquid nitrogen, lyophilized, and weighed. In most experiments, endothelial cells were removed 15 minutes after prelabeling with ³H-adenosine; however, in one experimental series, the intervening incubation time was increased to 60, 120, and 240 minutes.

In a series of preliminary experiments in which 0.75% DCA was perfused for 60 seconds to remove endothelial cells, no difference was seen on scanning and transmission electron microscopy compared with vessels treated for 15 seconds. However, removal of radioactivity during the first minute after DCA perfusion averaged 79±5% (n=3) and
94±0.5% (n=3) of total aortic radioactivity for 15- and 60-second DCA treatments, respectively (p<0.025); ATP contents were 20% lower in aortas treated for 60 seconds (p<0.01). Treatment of other vessels with DCA for only 8 seconds resulted in delayed washout of radioactivity.

### Analysis of Radioactivity and Adenine Nucleotides

Total radioactivity in the endothelial cell compartment was determined by collection of the effluent perfusate during the period of DCA perfusion and 3-minute washout. The volume of effluent was measured and an aliquot was counted for radioactivity by means of a liquid scintillation counter. To determine radioactivity sequestration in the vascular smooth muscle compartment, aortas that had been lyophilized after endothelial removal were dissolved overnight in 1 ml Soluene-100 (Packard Instruments, Downers Grove, Illinois) at 4°C and counted. Radioactivity values were corrected for different quenching of the two media by use of an internal tritium standard. Total aortic radioactivity was calculated as the sum of the two cellular compartments.

Specific radioactivity of adenine nucleotides was measured with a modification of a previously described high-pressure liquid-chromatography (HPLC) technique. Briefly, adenine nucleotides were extracted from lyophilized aortas in ice-cold perchloric acid (0.5 M) by use of a mechanical tissue homogenizer set at the highest speed for 2 minutes. After centrifugation at 15,000 rpm for 20 minutes at 4°C, the supernatant was neutralized with potassium hydroxide, lyophilized, and resuspended in 0.2-0.3 ml water. Samples were partitioned on a Waters 10-μm strong anion-exchange column (Waters Chromatography Div, Millipore, Milford, Massachusetts) using a nonlinear buffer gradient (Solution A: 7 mM KH₂PO₄, 6.7 mM KCl, pH 4.0; Solution B: 250 mM KH₂PO₄, 500 mM KCl, pH 5.0; flow rate 2 ml/min), and the column eluate was monitored at 254 nm. AMP was identified by internal standards and was quantified by peak height. ATP and ADP were identified by the retention times of external standards and were quantified by peak height. The respective eluate peak fractions were collected, counted for radioactivity, and corrected for radioactivity levels in fractions eluting before and after the respective adenine nucleotide peaks.

A different HPLC technique was used for analysis of the specific radioactivity of adenine nucleotides in aortas prelabeled with ³H-inosine. Samples were partitioned on a Waters C-18 column (5-μm) by use of a 0–20% nonlinear gradient (Solution A: 11 mM tetrabutylammonium hydrogen sulfate, 61 mM KH₂PO₄, pH 5.8; Solution B: 100% acetonitrile; flow rate 1.5 ml/min). This technique also permitted analysis of inosine, hypoxanthine, and inosine 5’-monophosphate (IMP).

### Microscopy

Aortas were prepared as described above and after 30 minutes of in vitro perfusion were fixed by perfusion with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 10 minutes at a rate of 2 ml/min with outflow pressure maintained at 40 cm H₂O. Vessels were fixed both with intact endothelium and after endothelial removal with DCA perfusion for 15 or 60 seconds. Aortas were also cannulated and perfusion fixed for 10 minutes in situ against an outflow pressure of 40 cm H₂O without further preparation. Vessels were cut longitudinally, mounted on cork plates, and immersed in fixative for at least 24 hours at 4°C. Half of each artery was prepared for scanning electron microscopy by critical point drying in CO₂, followed by sputter coating with palladium gold. The entire section was examined with a Philips SEM 515 scanning electron microscope (Philips Electronic Instruments, Mahwah, New Jersey). From the other half of each artery, three small blocks were cut from near the two ends and the middle. Semithin and ultrathin sections were obtained by standard procedures for light and transmission electron microscopy.

### Aorta Diameter

For determination of the effect of DCA treatment on aortic smooth muscle contractile function, aortic diameter was measured with a pair of piezoelectric crystals mounted on the central region of vessels prepared as described above. The external perfusion chamber was not used in these experiments. Ultrasonic pulses (4.5 MHz, repeat interval 1 msec) were used for measurement of the crystal transit time, which was recorded and calibrated with a specially designed micrometer. Vessels were constricted with an infusion of norepinephrine (0.1 μM), and the effect of an infusion of acetylcholine (0.3 μM) was determined. After a 60-second perfusion with 0.75% DCA and a 60-minute recovery period, the effects of norepinephrine and acetylcholine were again determined.

### Statistics

Differences between sample means were judged to be significant when evaluation by Student’s t test for unpaired data demonstrated p<0.05. Paired comparisons between means have been noted. Results are reported as mean±SEM.

### Results

**Microscopy**

The media of the aorta had an average thickness of 86.6±1.2 μm (mean±SEM, n=18 observations from three vessels) and was composed of six to eight layers of smooth muscle cells separated by elastic plates (Figure 1C). The endothelial cell layer of in situ fixed aortas was separated from the media by the internal elastic lamina, a basal membrane, and, in places, a small layer of myointimal cells.
Endothelial cell thickness inside and outside the nuclear area averaged 1.01±0.23 and 0.40±0.12 μm (n=21 determinations for each average), or 1.2% and 0.5% of the medial thickness, respectively. An uninterrupted endothelial cell layer was present in all vessels fixed in situ and in vitro (Figure 1A), and endothelial ultrastructure appeared normal except for the presence of cytoplasmic vacuoles in some cells in preparations fixed in vitro. In vessels fixed after 15 seconds of DCA perfusion (Figure 1B), no endothelial cells could be found with scanning and transmission electron microscopy. In semithin sections of vessels treated with DCA, all smooth muscle cell layers were present, although toluidine blue staining was less intense in the innermost layers. In ultrathin sections of DCA-treated vessels, smooth muscle ultrastructure of all layers appeared normal, except for vacuolated mitochondria observed occasionally in the innermost layer.

**Aorta Diameter**

Under control conditions, norepinephrine (100 nM) reduced the diameter of isolated, perfused aortas (flow rate 2 ml/min, pressure 60 cm H₂O) by an average of 29.7±9.2 μm (mean±SEM, n=4) from a control value of 1.02±0.09 mm; this change was fully reversed by infusion of acetylcholine (0.3 μM). Sixty minutes after DCA treatment, norepi-
Adenosine sequestration by endothelium-denuded intima in aortas that were denuded of endothelium by otherwise identical conditions (Figure 3, left panel). DCA treatment 30 minutes before prelabeling under conduct for measurement of adenosine sequestration during prelabeling. Parallel experiments were also conducted for measurement of adenosine sequestration in aortas that were denuded of endothelium by DCA treatment 30 minutes before prelabeling under otherwise identical conditions (Figure 3, left panel). Adenosine sequestration by endothelium-denuded vessels was less than half that by endothelium-intact vessels at adenosine concentrations of =1 μM (p<0.005). However, at higher adenosine concentrations (10 and 100 μM), this difference disappeared.

Aortas prelabeled with intact endothelium were further analyzed by use of the radioactivity removed with DCA to represent the endothelial compartment and the radioactivity remaining in the vessel to represent the smooth muscle compartment (Figure 3, right panel). Adenosine sequestration by the endothelial compartment (EC) increased steeply up to an adenosine concentration of 1 μM but became flatter above 10 μM. Adenosine sequestration in the smooth muscle compartment (SM) increased as adenosine concentration was raised. Adenosine sequestration in the smooth muscle compartment was only 8% of that in overlying endothelium at 12 nM adenosine; however, at 100 μM adenosine, sequestration by smooth muscle was twice as large as that by endothelium.

For determination of whether the sequestration of adenosine in the experiments reported above was mediated by phosphorylation or deamination of adenosine, additional experiments were carried out in which adenosine deaminase was inhibited by erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA). EHNA (5 μM throughout the experiment) had no significant effect on adenosine sequestration in endothelium-intact aortas at an adenosine concentration of 0.1 μM (Table 1). In contrast, at 100 μM adenosine, EHNA reduced adenosine sequestration in the endothelial compartment by 49% (p<0.005) and in the smooth muscle compartment by 10% (p<0.025) compared with separate controls without EHNA.

**Endothelial Removal**

After prelabeling of perfused rat aortas with [3H]-adenosine (12 nM) for 10 minutes followed by a 15-minute washout period, endothelial cells were removed by a 15-second perfusion interval with 0.75% DCA, and the washout of radioactivity into the effluent perfusate was determined in serial 30-second fractions. Of the total radioactivity sequestered during prelabeling, only 0.1%/min was released into the effluent perfusate at the end of the washout period before DCA treatment; 78.6±4.6% (n=3) of the radioactivity was released during the first minute after DCA treatment, and a total of 89.5±1.4% was released by the end of the third minute.

**Sequestration of Adenosine and Inosine**

The selectivity of adenosine incorporation by the vascular endothelium of perfused, endothelium-intact aortas was determined by prelabeling with [3H]-adenosine for 10 minutes at different adenosine concentrations (12 nM-100 μM). Radioactivity washed out of the aorta during DCA treatment (endothelial compartment) and radioactivity remaining within the vessel media after the 3-minute washout period (smooth muscle compartment) were both measured directly. In the concentration range 12 nM-1 μM, more than 90% of total radioactivity taken up by the aorta (endothelial compartment plus smooth muscle compartment) was trapped in the endothelium (Figure 2). At an adenosine concentration of 10 μM, fractional uptake of adenosine into the endothelium was significantly less (p<0.005), and at 100 μM adenosine, endothelial sequestration was only 33±4% (n=3) of the total. Less than 1% of the total radioactivity used for prelabeling was incorporated by aortas.

Data on the rate of adenosine sequestration into endothelium-intact aortas are shown in Figure 3 (left panel). Sequestration was calculated from the total incorporation of radioactivity in both cellular compartments and the specific radioactivity used during prelabeling. Parallel experiments were also conducted for measurement of adenosine sequestration in aortas that were denuded of endothelium by DCA treatment 30 minutes before prelabeling under otherwise identical conditions (Figure 3, left panel). Adenosine sequestration by endothelium-denuded vessels was less than half that by endothelium-intact vessels at adenosine concentrations of =1 μM (p<0.005). However, at higher adenosine concentrations (10 and 100 μM), this difference disappeared.

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Additional experiments were carried out that used 3H-inosine for prelabeling of intact aortas under otherwise identical conditions. The results (Table 2) indicated that the total sequestration of inosine in both endothelial and smooth muscle compartments was less than that of adenosine at all concentrations tested. Nevertheless, the data shown in Figure 2 indicate that at a concentration of 0.1 μM, the fractional incorporation of inosine into the endothelium (85%) was nearly as great as that of adenosine (93%).

Adenine Nucleotides

The intracellular fate of adenosine and inosine sequestered by aortas was determined by measurement of the distribution of radioactivity among the different adenine nucleotides. When 3H-adenosine (12 nM) was used for prelabeling of both endothelium-intact and endothelium-denuded aortas, between 42% and 62% of the total radioactivity incorporated into the adenine nucleotides was associated with ATP (Table 3). However, the specific radioactivity of AMP was higher than that of ATP (∼0.05, paired t-test) in the two (EC+SM and SM) intact aorta groups. Endothelial removal after prelabeling reduced the total radioactivity associated with adenine nucleotides by 90% (p<0.005). Smooth muscle of endothelium-denuded aortas exhibited a 3.3-fold (p<0.005) higher incorporation of radioactivity into adenine nucleotides than smooth muscle prelabeled with intact endothelium. Radioactivity incorporated into adenine nucleotides accounted for 70% (intact), 95% (SM), and 48% (denuded) of the total radioactivity sequestered by 3H-adenosine-prelabeled aortas reported in Figure 3. 3H-inosine prelabeling under identical conditions produced a pattern of distribution of radioactivity among the adenine nucleotides that was similar to that obtained with 3H-adenosine (Table 3). However, it is clear that adenosine was incorporated into adenine nucleotides more effectively than was inosine, since specific radioactivity values for total adenine nucleotides were 8–16 times higher (p<0.005) in aortas prelabeled with 3H-adenosine.

After prelabeling with 3H-inosine and washout for 15 minutes, radioactivity associated with hypoxanthine and IMP accounted for 5.6% and 1.0%, respectively, of total adenine nucleotide radioactivity; no radioactive inosine could be detected. Absolute levels of inosine, hypoxanthine, and IMP were below the detection limit of the HPLC system.

The ATP content of endothelium-denuded aortas (Table 3) was 68% (p<0.005) of the ATP content (1,430±60 nmol/g aorta dry, n=8) of aortas possessing endothelium. Thus, removal of endothelium with DCA resulted in a difference of 450 nmol/g. Significant but smaller differences in ADP and AMP content were also observed.

### Table 1. Effect of Inhibition of Adenosine Desaminase With EHNA (5 μM) on 3H-Adenosine Incorporation Into Endothelium-Intact Aortas

<table>
<thead>
<tr>
<th>Adenosine concentration (μM)</th>
<th>Incorporation into endothelial compartment (nmol/10 min/g aorta dry)</th>
<th>Incorporation into smooth muscle compartment (nmol/10 min/g aorta dry)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>Control (n=3) 3.0±0.3 140±8</td>
<td>Control (n=3) 0.46±0.09 440±5</td>
</tr>
<tr>
<td></td>
<td>EHNA (n=3) 2.7±0.2 72±10*</td>
<td>EHNA (n=3) 0.31±0.04 390±15</td>
</tr>
</tbody>
</table>

EHNA, erythro-9-(2-hydroxy-3-nonyl) adenine; n, number of experiments.

*p<0.005 vs. corresponding control.

f*p<0.025 vs. corresponding control.
Transfer of Purines

Further experiments were conducted for estimation of the transfer rate of radioactivity from endothelial adenosine nucleotides to smooth muscle adenosine nucleotides. For this purpose, endothelium-intact, 3H-adenosine–prelabeled aortas were incubated under resting conditions for 15, 60, 120, and 240 minutes before endothelial removal with DCA, and the radioactivity incorporated into smooth muscle ATP and ADP was determined. Aortic smooth muscle content of ATP and ADP appeared essentially constant for up to 4 hours of perfusion, except for lower values observed after 2 hours (Table 4). Radioactivity incorporated into smooth muscle ATP and ADP also appeared relatively stable at 3–8% of the total radioactivity sequestered in the endothelial compartment, except for lower values after 2 hours. The specific radioactivities of both ATP and ADP were significantly greater after 4 hours of perfusion than after 15 minutes.

Discussion

Perfused Rat Aorta

Brief perfusion of the isolated aorta with DCA proved to be an efficient means for the selective removal of vascular endothelium. The time of exposure and concentration of DCA used in the present study (15 seconds and 0.75%, respectively) were determined in a series of preliminary experiments to be the gentlest exposure at which complete endothelial removal was achieved (see “Materials and Methods”). Selectivity and completeness of endothelial cell removal were important for the interpretation of the reported biochemical measurements and, therefore, were carefully controlled: 90–95% of total radioactivity was washed out of aortas prelabeled with 3H-adenosine at low concentrations, and no endothelial cells were found in aortas with scanning electron microscopy. Furthermore, measurements of aorta diameter showed that the dilator effect of acetylcholine, mediated by an endothelium-dependent mechanism, was completely abolished. Selectivity of the effect of DCA was based on microscopic findings that showed no loss of smooth muscle lamina due to DCA. Normal smooth muscle function was demonstrated by enhancement of the constrictor response elicited by norepinephrine in DCA-denuded aortas, as has been reported after mechanical removal of the endothelium in the in vivo dog iliac artery and isolated rings of rat aorta. DCA (1.5%) was first used to remove the epithelium of isolated renal proximal tubules while leaving the basement membrane intact. DCA (1.5% for 10 minutes) has also been used to remove the vascular endothelium from short segments of the perfused rabbit aorta for determination of electrical conductivity of the endothelium. It should be noted that in the present study, DCA was used at a lower concentration and for a shorter perfusion interval.

The experimental model used in the present study provides the unique opportunity to investigate adenosine metabolism in the two main cellular compartments of the vascular wall in their in vivo anatomic relation. In addition, smooth muscle contractile function can be assessed simultaneously. In contrast with many previous studies that used the arterial ring and strip, the isolated perfused aorta (length 3.5 cm, diameter 2 mm) provides sufficient material for the analysis of the tissue content of adenosine nucleotides.

Endothelial Barrier

The results of the present study demonstrated that the vascular endothelium constitutes a metabolic barrier for the transport of adenosine in the arterial wall and accounts for more than 90% of total 3H-adenosine sequestration in the aorta at concentrations of ≤1 μM. These findings extended the recent observation that about 56% of the adenosine perfused through the isolated guinea pig heart at a concentration of 1 μM was trapped by the coronary endothelium. Evidently, arterial endothelium represents an even more effective barrier for adenosine transport than microvascular endothelium. In the present study, it was also found that aortic endothelium represents a barrier for inosine transport, although the barrier is less effective than for adenosine. A number of recent studies that used the multiple-indicator dilution technique for investigation of adenosine transport and metabolism have concluded that microvascular endothelium plays a crucial role in tissue uptake and sequestration of adenosine. Because these studies and others demonstrated that adenosine is predominantly incorporated into endothelial adenine nucleotides, the enzyme adenosine kinase appeared to be primarily responsible for the endothelial metabolic barrier for

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**Table 2. Incorporation of 3H-Inosine Into Endothelium and Smooth Muscle of Endothelium-Intact Rat Aortas**

<table>
<thead>
<tr>
<th>Inosine concentration (μM)</th>
<th>0.1</th>
<th>1.0</th>
<th>10</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incorporation into endothelial compartment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inosine incorporation (nmol/10 min/g aorta dry)</td>
<td>0.73</td>
<td>2.7</td>
<td>13</td>
<td>85</td>
</tr>
<tr>
<td>Inosine/adenosine incorporation×100 (%)</td>
<td>23</td>
<td>11</td>
<td>11</td>
<td>44</td>
</tr>
<tr>
<td>Incorporation into smooth muscle compartment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inosine incorporation (nmol/10 min/g aorta dry)</td>
<td>0.13</td>
<td>1.3</td>
<td>16</td>
<td>130</td>
</tr>
<tr>
<td>Inosine/adenosine incorporation×100 (%)</td>
<td>59</td>
<td>48</td>
<td>34</td>
<td>34</td>
</tr>
<tr>
<td>Fractional incorporation into endothelium (%)</td>
<td>85</td>
<td>67</td>
<td>45</td>
<td>39</td>
</tr>
</tbody>
</table>

Values are means from two experiments at each concentration. Average difference between duplicates was 28% of mean value. Inosine incorporation is shown as percent of adenosine incorporation at same substrate concentration (adenosine data from Figure 2).
Transported from endothelial cells to underlying mechanisms. Results obtained in the present study permit an intact, either by transport through endothelial cells or by salvage mechanisms. Finally, adenosine within the tide pool may have reached the subendothelial space and entered smooth muscle cells, where the adenosine nucleotide was returned to the adenine nucleotide. Adenosine nucleotides; EC, endothelial compartment; SM, smooth muscle compartment. Data shown are mean ± SEM; n = number of experiments per group.

### Transendothelial Transport and Metabolism

As is schematically outlined in Figure 4, at least three distinct processes may have played a role in the transendothelial transport of adenosine in the present experiments. One possibility is that adenosine taken up by the endothelium was phosphorylated and the resulting nucleotides were transported to underlying smooth muscle cells via gap junctions. A second possibility is that adenosine within endothelial cells was converted to inosine and hypoxanthine, which diffused across the subendothelial space and entered smooth muscle cells, where the purine moiety was returned to the adenine nucleotide pool via salvage mechanisms. Finally, adenosine may have reached the subendothelial space intact, either by transport through endothelial cells or by means of interendothelial clefts. The results obtained in the present study permit an estimate of the relative importance of these three mechanisms.

The possibility that adenosine nucleotides were transported from endothelial cells to underlying smooth muscle cells via gap junctions is suggested by the finding that the rat aorta contains relatively abundant myoendothelial junctions. Moreover, gap junction–mediated transport of radiolabeled uridine derivatives from endothelial cells to smooth muscle cells was extensive after 3–4 hours of incubation in coculture experiments. In the present experiments, endothelial radioactivity was transferred to underlying smooth muscle adenine nucleotides at a rate of not more than 1%/hr, based on the results shown in Table 4. Thus, gap junction–mediated transport of adenine nucleotides does not appear to play a major role in the intact aorta. It has recently been suggested that gap junction–mediated transport of cyclic AMP from endothelial cells to smooth muscle cells might explain the parallel activation of endothelial adenylate cyclase and increases in coronary flow during adenosine stimulation in guinea pig hearts. Based on the findings of the present study, such a mechanism is only plausible if gap junction permeability for cyclic AMP is substantially higher than for ATP. Alternatively, gap junctions may be more numerous in the microvasculature.

In a series of recent studies, intracoronary infusion of radiolabeled adenosine at a concentration of approximately 10 nM has been used to selectively prelabel adenine nucleotides of coronary endothelium in guinea pig hearts. However, it was uncertain if the entire vascular compartment (endothelium and smooth muscle) was labeled by this procedure. Assuming that the results of the present study can be applied to coronary arterioles, it...
appears that this prelabeling technique is selective for the coronary endothelium.

Another possible route for transendothelial transport of adenosine in the present study involves the formation of inosine and hypoxanthine in the endothelium and the sequestration of these adenosine products by smooth muscle. However, results from experiments in which EHNA was used to block formation of inosine from adenosine (Table 1) indicate that this pathway was of only minor importance for smooth muscle. In the experiments with \(^{3}H\)-inosine, incorporation of radioactivity into aortic adenine nucleotides most likely proceeded by the obligatory formation of hypoxanthine and subsequent phosphoribosylation to IMP (see Figure 4). Radiolabeled hypoxanthine was found in these aortas, but little IMP was present, suggesting that phosphoribosylation of hypoxanthine was the rate-limiting step. Direct phosphorylation of inosine is unlikely since inosine kinase activity is not normally present in mammalian tissue.\(^{24}\)

In isolated cardiomyocytes, incorporation of inosine into ATP was reported to be less than 0.08% that of adenosine.\(^{24}\) In contrast, in endothelium-intact aortas in the present study, inosine incorporation into ATP was 14% that of adenosine, 175 times greater than in isolated cardiomyocytes. These findings suggest the presence of a two-tiered purine salvage system in the endothelium. A high-affinity tier, mediated by adenosine kinase, appears important for the salvage of adenosine at low concentrations. A second, low-affinity tier apparently salvages inosine and hypoxanthine. This second tier may be important even under normal conditions, since endothelial cells in vivo appear to be exposed to higher concentrations of inosine and hypoxan-

thine than of adenosine.\(^{25}\) The findings obtained by use of EHNA to block adenosine deaminase (Table 1) suggest that under conditions where endothelial adenosine kinase approaches saturation, endothelial adenosine deaminase serves to shunt adenosine from the high-affinity to the low-affinity purine salvage pathway. Thus, endothelial adenosine deaminase and nucleoside phosphorylase\(^{26}\) can extend the effective concentration range over which adenosine can be salvaged.

The ATP content of endothelium-intact aortas averaged 1.4 \(\mu\)mol/g aorta dry, which is equivalent to 0.28 \(\mu\)mol/g aorta wet, assuming a wet/dry weight ratio of 5. This wet weight value is similar to that reported in rabbit aortas (0.3 \(\mu\)mol/g wet).\(^{27}\) An estimate can be made of the ATP content of the intact aortic endothelium in the present experiments by use of the loss of ATP during endothelial removal with DCA (0.45 \(\mu\)mol ATP/g aorta dry), the measured endothelial cell thickness (0.4 \(\mu\)m and 1.0 \(\mu\)m in extranuclear and nuclear regions, respectively), and vessel geometry (luminal surface area 290 \(cm^2/g\) aorta dry). Assuming that endothelial cell density equals 1.0 g/cm\(^3\), the calculated values for endothelial cell ATP content in vivo, based on extranuclear and nuclear dimensions, are 39 and 15 \(\mu\)mol/g EC, respectively. These values are similar to those reported for coronary microvascular endothelium in culture (12 \(\mu\)mol/g EC wet)\(^{17}\) but are about 100 times higher than those of vascular smooth muscle. Should DCA have damaged smooth muscle cells, resulting in ATP leakage, calculated endothelial cell content of ATP would be too high. This possibility appears rather unlikely, based on evidence discussed above and the finding that aortic smooth muscle ATP is comparatively low. It remains
an intriguing question, however, whether the high endothelial ATP content corresponds to a similarly high energy turnover. Indeed, the metabolic requirements of endothelial cells for ATP are not known.

Another possible explanation for smooth muscle adenosine uptake in the present study is via dephosphorylated adenosine nucleotides released during endothelial cell lysis caused by DCA treatment. Because of the high flow rate (6 ml/min) and the relatively low specific radioactivity of aortic adenine nucleotides (3.8 cpm/pmol), it can be calculated that this mechanism caused only a negligible overestimate of smooth muscle adenosine uptake.

**Endothelial Adenosine Gradient**

From the foregoing discussion, it is evident that in the present experiments the sequestration of radioactivity by smooth muscle underlying intact endothelium occurred predominantly via the uptake of labeled adenosine itself. The concentration of adenosine in the subendothelial space during prelabeling was less than that in the vessel lumen because of adenosine uptake and metabolism by endothelial and smooth muscle cells. However, the large fraction of adenosine trapped by the endothelium does not necessarily reflect the endothelial concentration gradient for adenosine. The subendothelial concentration of adenosine must be determined by at least three factors: 1) the permeability of the endothelial layer for adenosine, 2) the kinetic parameters for adenosine uptake from the subendothelial space by endothelial cells, and 3) the kinetic parameters for adenosine uptake by smooth muscle cells. For calculation of subendothelial adenosine concentration, aortas denuded of endothelium before prelabeling were used to generate a calibration curve (Figure 3, left panel) for smooth muscle adenosine uptake. This curve was then compared with the smooth muscle curve obtained by prelabeling of aortas with intact endothelium (Figure 3, right panel). Thus, the adenosine concentrations at equal levels of adenosine sequestration by smooth muscle of endothelium-intact and endothelium-denuded vessels provide a direct estimate of the adenosine concentration gradient across the intact endothelium. Subendothelial (abluminal) adenosine concentrations were estimated graphically at luminal adenine concentrations of 0.1, 1, 10, and 100 μM (Figure 5). Linear regression analysis of these data yielded the relation

$$\log [AR]_{\text{abl}} = 1.135 \times \log [AR]_L + 0.164$$

for luminal (L) and abluminal (abl) adenosine (AR) concentrations. This relation appears to be clearly different from identity. The error in this estimation was assessed by linear regression analysis of the logarithms of individual data from the denuded and intact smooth muscle preparations between 0.012 μM and 10 μM adenosine concentration. The 95% confidence intervals of the two lines were used for determination of minimum and maximum values for abluminal adenosine concentration; this range for abluminal adenosine concentration was equivalent to ±28% about the respective mean values (similar to those in Figure 5). In the calculation of abluminal adenosine concentration, it was assumed that the characteristics of smooth muscle adenosine uptake and sequestration were not altered by removal of the endothelial layer.

A major implication of these findings is that endothelial trapping of adenosine can lead to overestimates in the adenosine concentration gradient maintained by the endothelium. For example, while

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**FIGURE 4.** Schematic representation of pathways for adenosine transport and metabolism in endothelium-intact rat aorta. Dashed lines represent transport routes; solid lines represent enzyme-mediated changes. Adenosine transport both via endothelial cell cytoplasm and interendothelial clefts is indicated. VS, vascular space; EC, endothelial cell; SES, subendothelial space; SM, smooth muscle cell; ARs, adenosine in vascular space; AR, adenosine in subendothelial space; AN, adenine nucleotides; AR, adenosine; INO, inosine; HYPO, hypoxanthine; IMP, inosine 5'-monophosphate; 1, adenosine kinase; 2, adenosine deaminase; 3, purine nucleoside phosphorylase; 4, hypoxanthine guanine phosphoribosyltransferase; 5, adenylosuccinate synthetase; 6, adenylosuccinate lyase (adenylosuccinate not shown).

**FIGURE 5.** Endothelial concentration gradient for adenosine. Abluminal adenosine concentrations in endothelium-intact rat aorta were determined graphically at luminal adenosine concentrations of 0.1, 1, 10, and 100 μM using values in Figure 3 (see text). •—•, linear regression line for the four determinations; ———, line of identity.
93% of the adenosine was trapped by the endothelium at 0.1 μM, the calculated abluminal adenosine concentration was still 17% of the luminal concentration. In contrast, at a luminal adenosine concentration of 100 μM, when only one third of the adenosine was trapped by the endothelium, the abluminal concentration was 42% of the luminal concentration. Taken together, the endothelial barrier for adenosine is neither absolute at low concentrations nor completely overcome at high concentrations. If coronary arteriolar endothelium sustains similar adenosine concentration gradients, then endothelial mediation of the vasodilator action of adenosine need not be postulated. Throughout the adenosine coronary vasodilator concentration range (0.05-1 μM), smooth muscle adenosine concentrations during adenosine infusion should be 15–20% of the luminal concentrations. This implies that coronary arteriolar smooth muscle must be approximately five times more sensitive for adenosine than dose-response curves for infused adenosine indicate. Since microvascular endothelium appears to be more permeable for adenosine than aortic endothelium, this factor may be even less for resistance vessels.

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