# Adenosine transport and ectonucleotidase activity in pulmonary and aortic endothelial cells

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ABSTRACT

Circulating adenine nucleotides are catabolised by ectoenzymes at the endothelial cell surface. We have studied the characteristics of the ectoenzymes in two experimental systems: piglet aortic endothelial cells cultured in vitro, and the piglet isolated perfused lung. There are three separate ectonucleotidases (nucleoside triphosphatase, nucleoside diphosphatase, 5'-nucleotidase), which together form a system that sequentially degrades ATP→ADP→AMP→adenosine. Studies with phosphorothioate nucleotide analogues demonstrate that the ectoATPase and the ectoADPase each recognises a specific Mg(II)-nucleotide complex at its active site.

Adenosine is efficiently taken up from extracellular medium by cultured endothelial cells or on a single passage following bolus injection into the isolated pulmonary circulation. At low concentrations (∿1μM) metabolites are not released and the intracellular adenosine is predominantly converted to ATP. At high concentrations (∿100μM) products are released after intracellular deamination. Indicator dilution studies suggest that the pulmonary endothelial adenosine transporter has a Km of ∿35μM.

## INTRODUCTION

Extracellular adenine nucleotides and adenosine have potent actions on blood vessel tone and platelet function. ATP and ADP are dilators in many vascular beds, acting via specific P2 receptors on endothelium to induce smooth muscle relaxation as a consequence of the generation of the as yet poorly characterised endothelium-derived relaxating factor (1-4). Adenosine is also a dilator in several vascular beds, but acts directly on smooth muscle (5). ADP is a potent inducer of platelet aggregation and secretion, whereas ATP is a competitive antagonist of the

Carrier mediated transport of solutes from blood to tissue (D.L. Yudilevich and G.E. Mann, eds) Longman, London, 1985 (p213–222)

effects of ADP, and adenosine is an inhibitor of platelet activation however induced.

There are several potential sources of intravascular adenosine derivatives. Intracellular ATP concentrations are generally high (>5mM), so that any stimulus inducing sufficient changes in membrane permeability to small molecular weight intracellular components will, temporarily at least, cause local extracellular concentrations of adenosine derivatives to rise to levels where they are biologically active (the μM range). It was demonstrated over 30 years ago that the shock induced by severe trauma was likely to be due in large part to the release of ATP into the circulation (6). More recently, we have drawn attention to the fact that brief treatment with proteases can temporarily induce leakage of nucleotides from endothelial cells, in sufficient amounts to have biological actions, without permanently damaging the cells (7,8). Furthermore, both ADP and ATP are released from platelet granule stores (where they are present at concentrations of ∿1M) during platelet activation. Lastly, much experimental work supports the hypothesis that the release of adenosine from parenchymal cells as a result of ischemia or hypoxia forms one of the major signals for the subsequent reactive hyperemia (9).

Regulation of the intravascular concentrations of adenosine derivatives is therefore necessary to control the biological responses to these compounds. Although it was known in 1950 that ATP was efficiently inactivated on a single passage through the lung (10), it was primarily the work of the Ryans that established the presence of ectoenzymes at the surface of the pulmonary endothelial cell, which are responsible for the dephosphorylation of circulating nucleotides in vivo (11). Red cells can contribute to the removal of circulating adenosine (12,13) but its major fate in most species seems to be uptake by endothelial cells (14,15). We have studied the characteristics of the ectonucleotidases and of adenosine uptake in two experimental systems: piglet aortic endothelial cells cultured in vitro, and the piglet isolated perfused lung.

## ADENINE NUCLEOTIDE CATABOLISM IN THE PULMONARY BED

The extracellular catabolism of ADP or ATP, injected as a bolus (0.1ml) into the circulation of the piglet isolated lung, perfused (10ml/min) with Krebs' solution containing Ficoll 70 as an oncotic agent (16), is shown in Fig. 1. At the lowest doses there is almost complete conversion of ATP or ADP to adenosine during the single passage. With higher doses there is a progressive increment in the proportion of AMP, then ADP, and finally ATP in the venous effluent. The pattern of nucleotide concentrations found is consistent with the sequential degradation of ATP→ADP→AMP→adenosine, and the saturation, in turn, of the ability of the lung to catabolise

AMP, ADP and finally ATP. From this data in Fig.1, and analogous data obtained from bolus injections of AMP, concentration dependence curves for nucleotide catabolism were fitted (Fig. 2). The doses were expressed as mM concentrations by assuming distribution throughout an intravascular volume of 5ml, calculated from the mean transit time. The maximum capacity of the pulmonary circulation to catabolise nucleotides on single passage was calculated from these curves to be ∿1,16 and 30μmol/min per lung for AMP, ADP and ATP respectively.

## CHARACTERISATION OF ENDOTHELIAL ECTONUCLEOTIDASES

Sequential extracellular catabolism of ATP→ADP→AMP→adenosine also occurs when ATP is added to the medium above piglet aortic endothelial cells in culture (17). By performing experiments with various concentrations of individual nucleotides and measuring initial rates of catabolism we found Km values of 24, 155 and 475μM for AMP, ADP and ATP respectively, with $V_{max}$ values of 0.8, 9.2 and 64 fmol/min per cell (17,18). Since there are ∿$10^9$ endothelial cells in the piglet lung (derived from data in ref. 19), these $V_{max}$ values are very similar to the values reported above for the pulmonary bed.

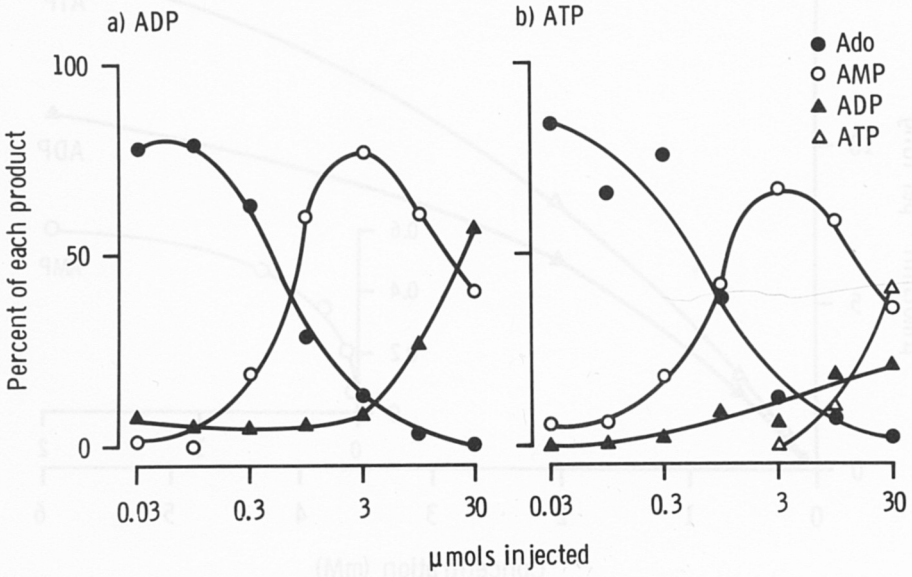


Figure 1. Extracellular catabolism of adenine nucleotides in the pulmonary bed. The patterns of catabolites obtained following bolus injection of various doses of [$^3$H]ADP and [$^3$H]ATP are shown. Metabolites were separated by t.l.c. Dipyridamole (10μM) was present to prevent uptake of adenosine (15).

By investigating a range of adenine nucleotide analogues as potential inhibitors or substrates for the ectonucleotidases we have been able to conclude that there are three distinct enzymes (nucleoside triphosphatase, nucleoside diphosphatase and 5'-nucleotidase) at the endothelial cell surface (17, 18, 20). Some of the main features of results from inhibitor studies are shown in Table 1. The triphosphatase is poorly inhibited by ADP or ADP analogues, whereas the diphosphatase is selectively inhibited by ATP analogues, and 5'-nucleotidase by ADP analogues. We tested nucleotide analogues in which the natural D-ribose moiety is replaced by L-ribose (18), and found that L-ATP and L-ADP are catabolised whereas L-AMP is not detectable converted to L-adenosine. These L-nucleotides are each competitive inhibitors of the catabolism of the corresponding D-nucleotide, but the relative affinities of the L- and D-isomers for each enzyme are very different (0.8, L- vs. D- ATP; 0.15, L- vs. D- AMP; 0.005, L- vs. D- AMP), further distinguishing the three enzymes by their ability to accept L-sugar substituted nucleotide at the active site.

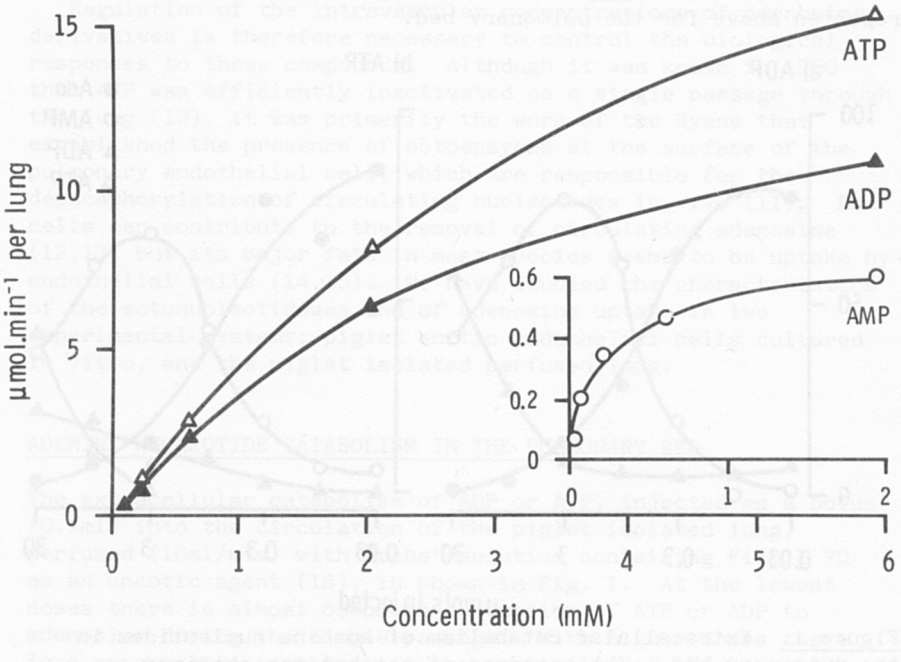


Figure 2. Concentration dependence of adenine nucleotide catabolism in the pulmonary circulation, derived from data similar to those shown in Fig. 1.

Table 1. INHIBITION OF ENDOTHELIAL ECTONUCLEOTIDASES

| Inhibitor | | Substrate | | |
|---|---|---|---|---|
| | | ATP | ADP | AMP |
| †AtetraP | $K_i$ | ≈1000 | 200 | |
| L-ATP | | 540 | 300 | |
| APPNP | | ≈ 500 | 30 | 10 |
| APPCP | | | 200 | |
| ATPγS | | | 30 | |
| ATPγF | | | 60 | |
| ATPβS(Sp) | | 1000 | <100 | |
| ATPβS(Rp) | | 50 | * | |
| ADP | | >3000 | | |
| APCP | | >>1000 | >>500 | <0.1 |
| A6P3 | | ≈1000 | 400 | no detectable inhibition |

†The table presents Ki values (μM) for the analogues listed. Abbreviations: AtetraP, adenosine 5'-tetraphosphate; APPNP, adenosine 5'-β,γ-imido-triphosphate; APPCP and APCP analogously abbreviated; ATPγS, adenosine 5'-(γ-thio)triphosphate; ATPγF and ATPβS analogously abbreviated; A6P3, 9-(6-hydroxyhex-1-yladenine)-6'-triphosphate.
* ATPβS(Rp), which is catabolised by the ectoATPase in the presence of $Mg^{2+}$, cannot be tested as an inhibitor of ectoADPase since it, like ATP, is a co-substrate with ADP for ecto-(nucleoside diphosphate kinase) (17).

Finally, we studied phosphorothioate nucleotide analogues such as ADPαS (18, 20). This compound has a sulphur atom substituted for a non-bridging oxygen on the α-phosphorus, and exists as a pair of diastereoisomers (Rp and Sp forms). In the presence of $Mg^{2+}$ (which preferentially binds to oxygen and not to sulphur) only one of these isomers (Sp) is catabolised (to AMPS) by the endothelial ecto-ADPase, suggesting that the enzyme recognises an Mg(II)-ADP complex. If $Cd^{2+}$ (which specifically binds to sulphur and not to oxygen) is used to replace $Mg^{2+}$, the apparent stereospecificity is reversed and the Rp isomer is selectively catabolised, which implies that the ecto-ADPase in vivo recognises a specific conformer of the α,β-bidentate Mg(II)-ADP complex. (For details of the reasoning leading to this conclusion, see refs. 20, 21). Similar experiments with the isomers of ATPβS led to an analogous result.

ENDOTHELIAL ADENOSINE UPTAKE

Adenosine is rapidly taken up from the extracellular medium above piglet aortic endothelial cells in culture and at low concentrations (∿1μM) almost entirely converted into intracellular adenine nucleotides, mainly ATP (22). In our original experiments we investigated the concentration dependence of adenosine uptake using incubation times of a few minutes, and found a high affinity process (Km = 3μM) that was blocked by dipyridamole. This Km value, however, is likely to represent the affinity of intracellular adenosine kinase and not that of the nucleoside transporter: as we have previously discussed (14, 23), adenosine concentrations equilibrate across the cell membrane in at most a few seconds, and studies of the kinetics of adenosine transport therefore require methods capable of following uptake over such a time scale.

Adenosine is also efficiently cleared from the circulation during single passage through the piglet isolated lung perfused with cell-free medium. We studied in detail the metabolism and fate of [$^3$H]-adenosine in this system (15). At low (1μM) concentrations overall uptake relative to an extracellular marker is about 50% (Fig. 3), and intracellular $^3$H is found in nucleotides, mainly ATP, as in the cultured

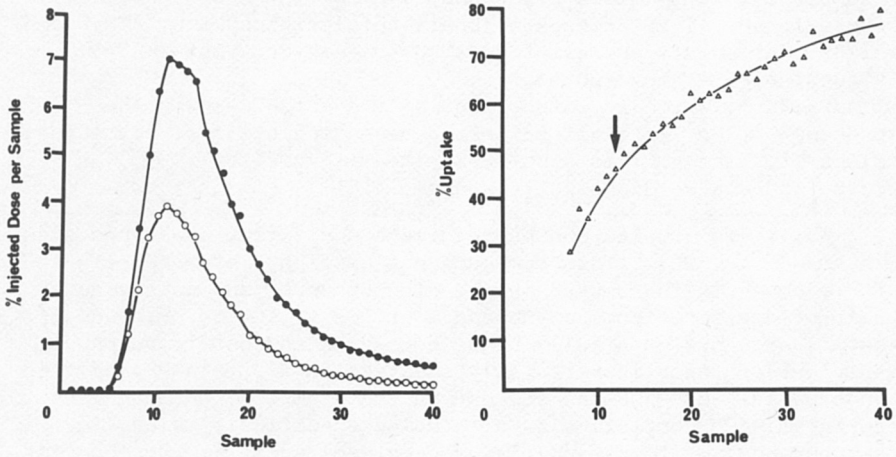


Figure 3. Cellular uptake of adenosine in the pulmonary bed. The left hand panel shows typical dilution curves for $^3$H and $^{14}$C after bolus injection of [$^3$H]-adenosine (open circles) and [$^{14}$C]-sucrose (an extracellular marker of very similar molecular weight) into the piglet isolated lung perfused with cell-free medium. The right hand panel shows a representative curve of the uptake of $^3$H relative to $^{14}$C; the arrow indicates the peak of the sucrose reference dilution curve.

aortic endothelial cells. With high concentrations of adenosine in the perfusate (100μM), inosine and hypoxanthine are released following intracellular adenosine metabolism, due to saturation of adenosine kinase (Km = 15μM in cell-free preparations from lung) before adenosine deaminase (Km = 450μM). Even with tracer doses of adenosine, however, a small amount of adenosine is deaminated leading to the presence of inosine in the venous effluent. Since uptake of this dose of adenosine can be completely blocked by dipyridamole (so that the adenosine and sucrose dilution curves are superimposed) without affecting the deamination, which can be blocked by deoxycoformycin, we conclude that there is a low activity of extracellular adenosine deaminase, perhaps an endothelial ectoenzyme, in the pulmonary bed (15). Although there is little evidence for such an enzyme in cultured aortic endothelial cells the activity is present on freshly isolated aortic endothelium (24), possibly indicating that it is not a true endothelial surface enzyme but one tightly bound from plasma.

Because the indicator dilution technique essentially monitors uptake over a period of a few seconds, and at least the initial part of each uptake curve is usually interpreted as measuring uptake across the first barrier (ie. the endothelial cell luminal plasma membrane), we felt it would be of interest to investigate the saturability of this uptake process. As shown in the right hand panel of Fig. 3, the uptake curve for a trace dose of adenosine relative to sucrose rises with time. Whether this relatively high late uptake should be interpreted in the manner suggested by Yudilevich and co-workers (see eg. 25 and refs therein) to represent an uptake process at a deeper barrier (eg. a parenchymal or epithelial cell membrane), or, as suggested by Bassingthwaighte (this volume) to include a preferential uptake of adenosine at the abluminal plasma membrane of the endothelial cell, is open to debate. Thus, although we constructed complete uptake curves with increasing concentrations of unlabelled adenosine (up to 100μM) added to the perfusate, we studied the way in which the uptake varied at the peak of the reference (sucrose) curve, and assumed that this reflects uptake predominantly at the luminal surface of the endothelium. Applying the Martin de Julian & Yudilevich equation to transform uptake into influx (26) we obtained an apparent Km value of 35μM and a Vmax of 19nmol/min per g wet lung tissue. This Km value is substantially different from the Km for adenosine kinase, and rather similar to estimates for the Km of the nucleoside transporter obtained from rapid uptake studies in other systems (12).

## CONCLUSIONS

Circulating adenine nucleotides are degraded in capillary beds by ectoenzymes at the endothelial cell surface. These enzymes

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