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Effects of pulmonary oedema on pharmacokinetics of adenosine in rat isolated lungs

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1 Pulmonary oedema, assessed by decreases in the lung dry weight:wet weight ratio, was induced in rats by a single i.p. injection of α-naphthylthiourea (ANTU). The oedema reached a peak at 4 h after ANTU and had completely resolved after 28 h.

2 Pulmonary pharmacokinetics of adenosine were measured in isolated, perfused lungs using radiolabelled adenosine and sucrose, injected into the perfusate as a single bolus.

3 By 1 h after ANTU the 1 min efflux of tracer for adenosine increased to over 60% and remained high until 16 h after ANTU. The time for 50% of injected radioactivity to appear in lung effluent ($t_{\frac{1}{2}}$) for adenosine was reduced from its normal value of $>$ 120 s to a minimum of 27 s at 1 h after ANTU. The proportion of adenosine in lung effluent did not change until 16 h after ANTU treatment but returned to normal by 50 h.

4 There were only minimal changes in the $T_{\frac{1}{2}}$ and 1 min efflux for sucrose following ANTU treatment.

5 It appears that both the uptake and metabolism of adenosine are affected by ANTU-induced lung damage. The early effects are chiefly on uptake with metabolism remaining normal. Later (after 16 h) metabolism is decreased with uptake recovering to normal levels.

6 The effects on adenosine uptake paralleled the development and the resolution of oedema, suggesting that this variable might provide a biochemical index of the physical processes leading to lung oedema.

Introduction

Pulmonary oedema induced *in vivo* by α-naphthylthiourea (ANTU) modifies the metabolism of 5-hydroxytryptamine (5-HT; Block & Schoen, 1981), prostaglandin E_2 (PGE_2; Bakhle, 1982), thromboxane B_2 (TxB_2; Bakhle & Grantham, 1985) and arachidonic acid (AA; Pankhania & Bakhle, 1985) in rat lung. One of the features of ANTU-induced pulmonary oedema is damage to the endothelium of the pulmonary capillaries (Cunningham & Hurley, 1972; Meyrick *et al.*, 1972). Adenosine is extensively metabolized on passage through the pulmonary circulation of rat isolated lung (Bakhle & Chelliah, 1983) most probably by the endothelial cells (Dieterle *et al.*, 1978; Pearson *et al.*, 1978). It was therefore reasonable to assume that ANTU-induced injury would affect adenosine metabolism in rat lung. The experiments described here set out to test this assumption. Some of these findings have been presented in a preliminary form to the Physiological Society (Bakhle & Grantham, 1986).

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Methods

Preparation of animals

Male rats (200–280 g) were injected intraperitoneally with 10 mg kg^{-1} bodyweight of ANTU suspended in olive oil (4 mg ml^{-1}). Sham-treated animals received the same volume of olive oil only. At the stated times after injection, the rats were anaesthetized with pentobarbitone (60 mg kg^{-1}) and the lungs removed, either for weighing or for whole organ perfusion.

Determination of lung weight

In a group of animals whose lungs were not used for perfusion studies, the thorax was opened carefully and any fluid present in the pleural cavity sucked out and weighed. The lungs were rinsed in 0.9% (w/v) saline, blotted dry and trimmed free of heart and other extraneous tissue. They were weighed immediately after removal and then again after drying to constant

weight. The lung dry:wet weight ratios were calculated from these measurements.

Preparation of perfused lungs and measurement of lung pharmacokinetics

Isolated lungs, perfused via the pulmonary artery with gassed (95% O_2, 5% CO_2), warmed (37°C), Krebs solution at 8 ml min^{-1} (Bakhle *et al.*, 1969) were used in the measurement of efflux kinetics for adenosine and an inert extracellular marker, sucrose. The Krebs solution contained indomethacin (3 $\mu g\,ml^{-1}$) to prevent any effects of endogenous prostaglandins on lung pharmacokinetics. Bolus injections (0.1 ml) of combined radioactive substrates (10 nmol, 50 nCi ^{3}H-labelled adenosine and 1 nmol, 10 nCi ^{14}C-labelled sucrose) were given into the perfusate flow entering the lung.

The effluent perfusate was collected in 4 drop fractions (ca. 3 s) immediately before, during and after the injection for a total of 2 min. Radioactivity in each fraction was measured by liquid scintillation (see below). Two measures of efflux kinetics were determined: the $t_{\frac{1}{2}}$ value for the substrate which is the time taken for 50% of the injected radioactivity to emerge from the lung and the effluent radioactivity collected after 1 min (expressed as a percentage of the injected radioactivity).

Chromatographic analysis of lung metabolism

Lung effluent was collected in a single fraction for 1 min after injection of [^{3}H]-adenosine (10 nmol, 5 μCi) to measure metabolism of adenosine. Where this was done in lungs also used for pharmacokinetic studies, the two injections were made at least 10 min apart. This sample was immediately adjusted to pH 3.5 with 10% perchloric acid to prevent further breakdown of adenosine by enzymes in the lung effluent. An aliquot of the acidified effluent was taken to measure total radioactivity. Further 30 μl aliquots of lung effluent were applied to plastic-backed fluorescent silica gel thin layer chromatography (t.l.c.) plates to separate the metabolites of adenosine, as described by Bakhle & Chelliah (1983), using the solvent system *n*-butanol: ethyl acetate:methanol:ammonia, 7:4:3:7 (Shimizu *et al.*, 1970). On average, 72% of the radioactivity was recovered from the t.l.c. plates. The mixture of labelled and unlabelled adenosine used for injection gave the following distribution (expressed as a percentage of the total radioactivity on the t.l.c. plate), inosine 3.3 ± 0.7, hypoxanthine 8.7 ± 1.5, adenosine 82.3 ± 1.3 ($n = 13$).

Radioactivity in lung effluent or t.l.c. eluates was measured by mixing the sample with 10 vol. of Optiphase Safe (LKB) in plastic vials and counting in a Packard model 4640 spectrometer. Counts were corrected for quenching using external standard channels ratio and were converted to d.p.m.

Materials

ANTU was obtained from Eastmas Kodak, sodium pentobarbitone (Sagatal) from May and Baker Ltd, unlabelled adenosine and indomethacin from Sigma. The radiolabelled substrates, [2-^{3}H]-adenosine (21 Ci $mmol^{-1}$) and [U-^{14}C]-sucrose (555 mCi $mmol^{-1}$), were obtained from Amersham International and Optiphase Safe scintillant fluid from LKB. Unlabelled sucrose, all chemicals for Krebs solution and solvents for t.l.c. were of Analar grade and were obtained from BDH Chemicals Ltd. Plastic-backed t.l.c. plates of silica gel with fluorescent indicator (20 cm × 20 cm) were obtained from Merck.

Statistical methods

Results are expressed as mean values (± s.e.mean) from n experiments (lungs). Differences between means were tested for significance with the unpaired t test and values of $P < 0.05$ taken as significant.

Results

Physical variables

The development and resolution of pulmonary oedema were followed by measuring the pleural exudate and the lung dry:wet weight ratio (Table 1) at fixed times after the single injection of ANTU. One of the most reliable and the most usual measures of pulmonary oedema is the dry:wet weight ratio (Staub, 1974) and this variable showed a minimal value, i.e. maximal oedema, at 4 h. The oedema spontaneously resolved by 50 h after ANTU. Sham-treated rats, i.e., those treated with olive oil only, did not show changes in these variables, when compared with untreated rats (no measurable pleural exudate; dry:wet ratio of 20.0 ± 0.1, $n = 12$).

Pharmacokinetic variables

The metabolism of [^{3}H]-adenosine was first assessed by the kinetics of ^{3}H efflux from lung after a bolus injection of 10 nmol adenosine into the perfusate entering the pulmonary circulation. A typical efflux profile is shown in Figure 1 for adenosine and for sucrose in a lung from an untreated rat. The shape of the two efflux profiles is very similar but the peak height of the efflux of the adenosine tracer is less than half the height of that derived from sucrose. This difference is made more obvious in the cumulative efflux curves in Figure 1b. Over 80% of the injected

[^{14}C]-sucrose but less than 50% of the label derived from adenosine appears in effluent within the standard collection time of 40 fractions (about 2 min). The two variables derived from these curves are the $t_{\frac{1}{2}}$ (time for 50% of injected radioactivity to appear in the effluent) and the 1 min efflux (the cumulative radioactivity up to 1 min). In normal lung, only sucrose provides a $t_{\frac{1}{2}}$ value but both substrates yield a value for the 1 min efflux.

The effect of treatment *in vivo* with ANTU on the $t_{\frac{1}{2}}$ value for adenosine is illustrated in Figure 2. Very soon after ANTU, the $t_{\frac{1}{2}}$ value was dramatically reduced from > 120 s to 27 s. Low $t_{\frac{1}{2}}$ values continued until 6 h after ANTU and, by 28 h, the value had returned to normal, i.e. > 120 s. The $t_{\frac{1}{2}}$ and 1 min efflux values for both adenosine and sucrose after ANTU are summarized in Table 2. Sucrose efflux was only transiently affected by ANTU at 1 h, whereas the efflux of adenosine-derived ^{3}H showed earlier and more marked changes, with both variables showing a maximal effect between 1 and 6 h and returning to normal by 28 h.

The radioactivity leaving the lung is not all associated with adenosine and analysis of the effluent radioactivity by t.l.c. showed only about 40% to be unchanged adenosine under normal conditions (Table 3). At 1, 2 and 4 h after ANTU, the times at which the 1 min efflux was at its peak, the analysis showed only small changes in the proportion of inosine. The proportion of adenosine and its major metabolite, hypoxanthine did not change until 16 h after ANTU. The maximal change in the composition of the effluent occurred at 28 h, when the 1 min radioactive efflux was normal (Table 3). The metabolites in the effluent from sham-treated lungs did not differ from those for untreated lungs shown in Table 3.

In Figure 3, the composition of lung effluent has been expressed as the concentration of each component, i.e., allowing for the changes in the 1 min efflux of total radioactivity. For the first 4 h after ANTU, adenosine and its major metabolite, hypoxanthine, rose in parallel to a maximum of about 2.5 times the normal, untreated values. The rapid fall in metabolite concentration after 6 h was not accompanied by a fall in adenosine, even at 28 h when the metabolite concentrations were well below the normal values.

Also shown in this Figure is the time course of the oedema (as dry:wet lung weight ratio). The changes in effluent adenosine reached their maximum well in advance of maximum oedema and adenosine concentrations remained high between 6 and 28 h during the resolution of the oedema. Furthermore, the lower concentrations of hypoxanthine persisted well after the physical state of the lung had returned to normal. However, the 1 min efflux of total radioactivity returned towards normal over the same period.

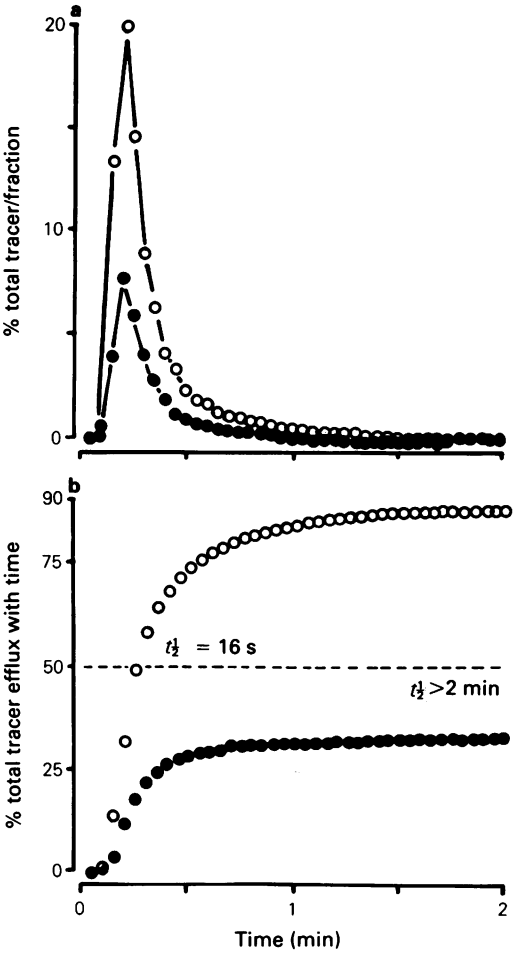

Figure 1 Efflux of radioactivity from rat isolated lung following a combined injection of [^{14}C]-sucrose (○) and [^{3}H]-adenosine (●). Lung effluent was collected in 4 drop fractions (approx 3 s) for 2 min after the radiolabel injection (10 nCi ^{14}C, 50 nCi ^{3}H) and the total radioactivity in each fraction measured. (a) Shows the radioactive efflux in each fraction from a normal lung. (b) Shows the cumulative efflux of ^{3}H and ^{14}C. The cumulative curves in (b) were constructed from the data in (a) and provide the two variables of lung pharmacokinetics for the radiolabels, i.e. the $t_{\frac{1}{2}}$ and 1 min efflux values, as shown.

Discussion

Our experiments showed that adenosine pharmacokinetics were affected by lung injury leading to oedema induced by ANTU. This effect would be compatible with the endothelial cell damage associated with this model of pulmonary oedema (Cunningham & Hurley, 1972; Meyrick *et al.*, 1972).

The loss of the normal permeability barrier provided by the endothelium which allowed increased extravascular fluid to accumulate did not, however, greatly affect the pharmacokinetics of sucrose. This substrate was used as a marker of extracellular space so that purely physical factors such as an increased volume of distribution available to solutes of this molecular weight range could be recognized. For instance, if the oedematous lung had provided an increased extracellular volume for sucrose to enter, then its efflux would have been slower and we would have expected a similar change in the efflux of tracer derived from adenosine (^{3}H). In fact, the ^{3}H- efflux was at least 400% faster in the early stages after ANTU. This might have been due to a reduction in the space available to adenosine, for instance because the injury had closed vessels normally perfused. Such a physical change should equally make sucrose efflux faster to the same extent but, in fact, the sucrose efflux was 20% faster only at 1 h after ANTU and then returned to normal. Furthermore, ^{3}H- efflux returned to normal by 28 h after ANTU and sucrose efflux was still unchanged. We are therefore confident that physical changes in extracellular space were not the most important factors in the changes in adenosine pharmacokinetics.

The ^{3}H- efflux is not equivalent to adenosine efflux because the radioactivity in the lung effluent derived from [^{3}H]-adenosine includes metabolites, chiefly hypoxanthine, as well as unchanged adenosine. These considerations illustrate another aspect of our results — the separate effects of lung injury on uptake and on

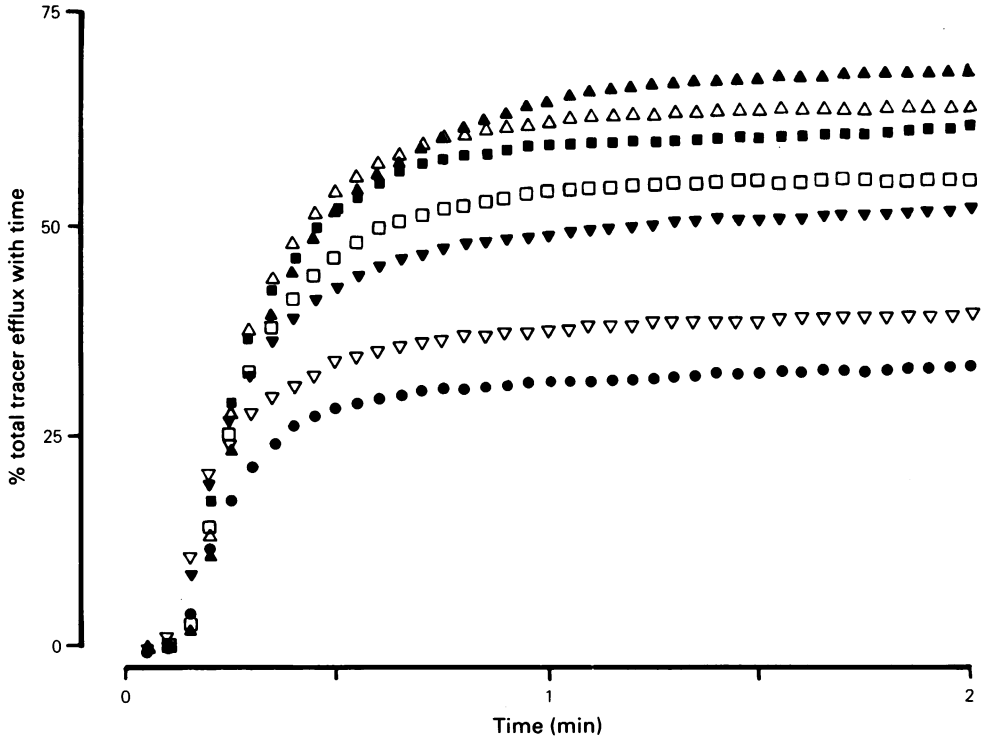

Figure 2 Effect of α-naphthylthiourea (ANTU) treatment on ^{3}H efflux from rat isolated lung after [^{3}H]-adenosine injection. A series of cumulative efflux curves is shown from individual lungs at 1 (■), 2 (△), 4 (▲), 6 (□), 16 (▼) and 28 (▽) h after ANTU treatment ((●) untreated lungs). There is a rapid decrease in the $t_{\frac{1}{2}}$ together with a corresponding increase in the 1 min efflux value for adenosine as early as 1 h after treatment. This alteration in adenosine pharmacokinetics persists until 4 h after treatment and then slowly returns towards normal over the next 24 h.

Table 1 Effects of α-naphthylthiourea (ANTU) treatment on the physical properties of rat lung

	Untreated	*Time after treatment* (h) *1*	*2*	*4*	*6*	*16*	*28*	*50*
Lung dry:wet weight ratio†	21.0 ± 0.7	19.0 ± 0.1*	17.6 ± 0.6*	16.0 ± 0.1*	16.4 ± 0.2*	17.7 ± 0.2*	19.0 ± 0.3*	19.7 ± 0.2
Weight of pleural transudate (g)	ND	ND	ND	2.7 ± 0.4	6.1 ± 0.6	4.3 ± 1.7	ND	ND
n	6	3	7	19	8	5	3	5

* Significantly different from value for untreated animals ($P < 0.05$, unpaired t test).
† These values are expressed as (lung dry weight ÷ lung wet weight) × 10^2
ND = not detectable.
The values are means ± s.e.mean of results obtained from the number of animals shown (*n*). Treated rats received a single i.p. injection of ANTU, suspended in olive oil (10 mg kg^{-1} bodyweight) and, at the time shown after this injection were anaesthetized with pentobarbitone and the lungs and transudate removed.

Table 2 Effect of α-naphthylthiourea (ANTU) treatment on efflux of radioactivity from rat isolated lung

		Untreated	*Time after ANTU treatment* (h) *1*	*2*	*4*	*6*	*16*	*28*
[^{3}H]-adenosine	$t_{\frac{1}{2}}$ (s)	116	27.2 ± 2.5	35.7 ± 6.2	29.0 ± 2.7	32.6 ± 6.6	60	123
	1 min efflux (% tracer recovery)	37.5 ± 2.2	61.8 ± 2.5*	60.0 ± 3.9*	60.5 ± 2.3*	60.3 ± 2.9*	44.3 ± 3.7	37.8 ± 0.9
[^{14}C]-sucrose	$t_{\frac{1}{2}}$ (s)	16.5 ± 0.7	13.8 ± 0.3*	16.9 ± 0.9	19.1 ± 1.9	17.1 ± 1.2	15.8 ± 0.3	14.8 ± 0.6
	1 min efflux (% tracer recovery)	81.5 ± 2.0	87.8 ± 1.3*	84.3 ± 3.2	79.6 ± 2.8	82.3 ± 2.6	82.2 ± 1.3	88.3 ± 2.1
n		5–7	4	4–5	4	4	4	4

*Significantly different from value for untreated rats ($P < 0.05$).
The values shown are the means ± s.e.mean of results obtained from the number of animals used (*n*). The two radiolabelled substrates were combined in a single injection and fractions of effluent collected as described in the Methods.

For [^{3}H]-adenosine, the $t_{\frac{1}{2}}$ values in untreated lungs and at 16 and 28 h after ANTU are given as the minimum values recorded; under these conditions, in most lungs the ^{3}H recovered did not exceed 50% within the total collection time (about 2 min).

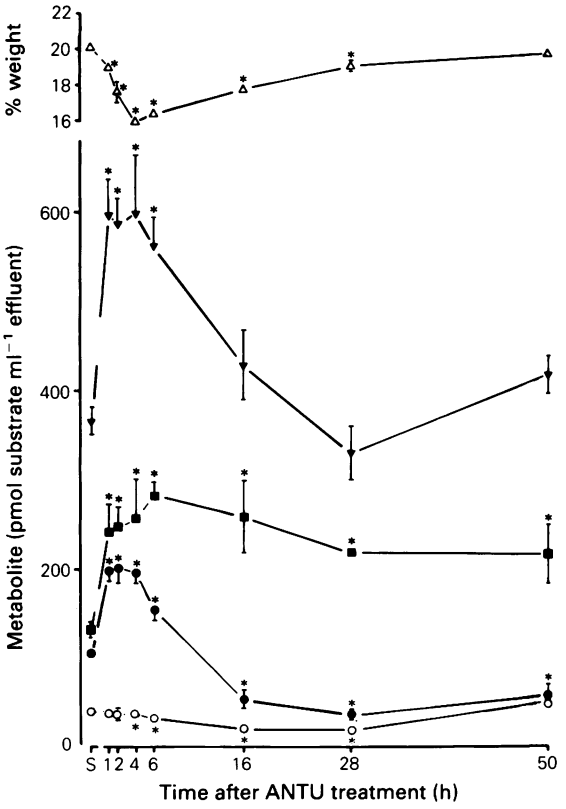

Figure 3 Adenosine metabolism during pulmonary oedema. The composition of the lung effluent has been expressed in terms of amount of metabolite per ml of lung effluent. Adenosine (■) and hypoxanthine (●) levels rise together as early as 1 h after α-naphthylthiourea (ANTU) treatment above their respective sham-treated (S) values. Adenosine remains elevated up to 50 h after ANTU treatment, while inosine (○) and hypoxanthine levels decrease to below their sham-treated values from 4 to 6 h respectively. The total amount of radioactivity (▼) in 1 ml of effluent increased in parallel with adenosine and hypoxanthine, returning to normal at 16 h after treatment. At the top of the figure is physical lung oedema, expressed as the lung dry:wet weight ratio (△). A small decrease in this parameter was apparent 1 h after treatment but the maximum change did not occur until 4–6 h after ANTU. Thereafter, the oedema slowly resolved until, at 50 h after treatment, the ratio fell within the normal range. *Significantly different ($P < 0.05$) from sham-treated values; $n > 3$. Vertical lines represent s.e.mean (absent when they fall within the symbol size).

metabolism. A simple inhibition of uptake would lead to a faster efflux but with a decreased proportion of metabolite (Bakhle & Chelliah, 1983) because the deaminase and nucleoside phosphorylases are intracellular enzymes (Rubio *et al.*, 1972). If these catabolic enzymes were inhibited but adenosine uptake was unchanged then we might expect the same efflux rate with decreased metabolites. If the kinases, which normally phosphorylate most of the adenosine taken up (Bakhle & Chelliah, 1983; Hellewell & Pearson, 1983), were inhibited, then the prediction would be for a greater proportion of metabolites and a normal efflux rate. What we observed was none of these possibilities. The faster efflux and increased amount of adenosine in effluent must derive from a decrease in uptake. However, the proportion of metabolites was normal, i.e. the amount of metabolite increased proportionately. This is not easy to explain if the catabolic enzymes are intracellular but, if there were an extracellular source of these enzymes, as has been suggested (Andy & Kornfeld, 1982; Hellewell & Pearson, 1983), then decreased uptake of adenosine could make more substrate available to extracellular deaminases and thus increase hypoxanthine levels in the lung effluent.

In the later stages, approximately at the peak of the oedema, the amounts and proportion of metabolites in the effluent fell, while the amounts of adenosine remained the same. These changes must reflect the loss of the catabolic enzyme activity. At this stage of oedema, the cellular damage is most obvious so that the loss of enzymic activity is not surprising. However, uptake of adenosine appeared to be functioning normally because, if it were not, a further increase in the amount of effluent adenosine, reflecting the impaired metabolism, would be expected at this time. Actually, the recovery in adenosine uptake seems to keep pace with the loss of metabolism and adenosine levels remain at the same level as during the early stages of injury. This uptake may be in cells other than endothelium – smooth muscle cells, fibroblasts (Dieterle *et al.*, 1978; Pearson *et al.*, 1978) – as the subendothelial layers of cells are exposed following the loss of endothelium.

The temporal correlation between oedema and adenosine pharmacokinetics shows clearly that biochemical derangement precedes, by several hours, the maximum physically detectable derangement of permeability. Although the oedema and efflux kinetics returned to normal over a similar period, the biochemical disturbance in terms of adenosine metabolites persisted for up to 22 h after the oedema had resolved. A similar prolonged biochemical disturbance is seen with PGE_2 and TxB_2 in the same model of pulmonary oedema (Bakhle, 1982; Bakhle & Grantham, 1985). Although endothelial cells lack the enzymes required to oxidize either substrate (Ody *et*

Table 3 Effect of α-naphthylthiourea (ANTU) treatment on adenosine metabolism in rat isolated lung

	Untreated	*Time after ANTU treatment* (h) *1*	*2*	*4*	*6*	*16*	*28*	*50*
Adenosine	44 ± 5	42 ± 1	44 ± 3	44 ± 3	53 ± 2	63 ± 5*	64 ± 2*	53 ± 5
Hypoxanthine	27 ± 4	36 ± 3	36 ± 3	35 ± 2	30 ± 1	15 ± 3*	12 ± 1*	16 ± 4
Inosine	12 ± 2	9 ± 1*	9 ± 1*	8 ± 1*	7 ± 0.3*	7 ± 1*	7 ± 1*	14 ± 1
n	4	4	4	4	4	4	4	3

* Significantly different from value for untreated rats ($P < 0.05$)
The values are means ± s.e.mean of results obtained from the number of animals shown (*n*). Lung effluent was collected in a single 1 min fraction after the injection of [^{3}H]-adenosine and analysed by t.l.c. Radioactivity associated with each of the marker compounds shown is expressed as a percentage of the total ^{3}H on the t.l.c. plate. The R_F values for the marker compounds were as follows: ATP and ADP, 0; AMP, 0.04; inosine, 0.35; hypoxanthine, 0.53; adenosine, 0.65; adenine, 0.76.

al., 1979; Ali *et al.*, 1980), both are less metabolized after ANTU treatment and the decreased metabolism persists up to 28 h after ANTU. Synthesis of prostacyclin (PGI_2) which is often associated with endothelial cells (Moncada & Vane, 1981), is actually increased in this model of endothelial cell injury (Pankhania & Bakhle, 1985), again with a longer duration than the measurable oedema. However, neither PGE_2 metabolism nor PGI_2 synthesis changed as early as adenosine pharmacokinetics, the earliest effects on the prostaglandins being seen at 4–6 h after ANTU.

Although ANTU-induced oedema has no direct clinical counterpart, it does share three features with the Adult Respiratory Distress Syndrome (ARDS) – high permeability oedema, endothelial cell damage and the involvement of oxygen-derived free radicals (Fox *et al.*, 1983). Furthermore, ANTU treatment increases the rate of clearance of ^{99}Tc-DTPA from the airways (Minty *et al.*, 1987), an effect also seen in patients with pulmonary oedema (Jones *et al.*, 1983). It may therefore be relevant to consider the pharmacodynamic effects of the metabolic changes described.

The pulmonary circulation has at least three systems with a net anti-aggregatory effect on platelets – inactivation of pro-aggregatory 5-HT and adenosine-5'-pyrophosphate (ADP), and formation of anti-aggregatory PGI_2. Inactivation of ADP leads to formation of adenosine, itself an anti-aggregatory substance.

Endothelial damage would increase the possibilities of intravascular platelet aggregation as subendothelial collagen is exposed. In this model (ANTU-induced oedema), there is a small reduction in 5-HT metabolism (Block & Schoen, 1981) which could have a pro-aggregatory effect. Against this, there is increased PGI_2 synthesis (Pankhania & Bakhle, 1985). For adenosine, the first change was to decrease uptake, i.e. increase intravascular levels. Even during the later stages of ANTU-induced lung injury, the amounts of adenosine in the effluent remained above normal levels. Thus the anti-aggregatory systems in the pulmonary circulation appear to be increased or at least maintained, in a condition where the stimuli for platelet aggregation would be increased. A further inference from our results would be that the changes in the metabolic function of the lung so far described in this model of pulmonary oedema appear to be protective responses to the original insult rather than causes or mediators of the injury.

Finally, one of the long-term aims of this study of pulmonary oedema is to find a biochemical index of lung injury which would give early warning of the physical state to come and provide a biochemical measure of the physical derangement. Adenosine pharmacokinetics appear to provide the best example so far of such an index. Whether or not adenosine will be an equally useful substrate in other models of acute lung injury remains to be established.

C.J.G. is an MRC scholar.

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