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Increased flow-induced ATP release from isolated vascular endothelial cells but not smooth muscle cells

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1 Freshly harvested smooth muscle cells and endothelial cells isolated from the rabbit aorta were perfused (0.5 ml min^{-1}) and stimulated twice by an increase of flow rate (3.0 ml min^{-1}) in order to compare their ability to release adenosine 5'-triphosphate (ATP).

2 In smooth muscle cells, the basal release of ATP (0.0265 ± 0.0033 pmol ml^{-1} per 10^6 cells) was not increased during periods of increased flow ($P = 0.2$).

3 In endothelial cells, the concentration of ATP in the perfusate during periods of low flow (0.0335 ± 0.0038 pmol ml^{-1} per 10^6 cells) was significantly increased by 14 times and 5 times during the first and second periods of increased flow, respectively.

4 The release of ATP by endothelial cells did not appear to be caused by the lysis of cells during the period of increased flow because it can be reproduced several times and because there was no difference between lactate dehydrogenase activity in perfused cells and that in non-perfused cells.

5 These results show that, of the two major cell types of the vascular wall, only endothelial cells react to shear stress by releasing ATP.

Keywords: ATP; aorta; endothelial cells; smooth muscle cells; ATP release

Introduction

Adenosine triphosphate (ATP) is a transmitter released upon nerve stimulation of a large variety of sympathetically innervated tissues, including blood vessels (Burnstock *et al.*, 1970; Su, 1975; White, 1988). Upon vascular wall stimulation, there is an overflow of ATP that includes release from both neuronal as well as extraneuronal sites (Levitt & Westfall, 1982; Westfall *et al.*, 1987). Although ATP is not taken up by endothelial cells and smooth muscle cells, these vascular cells incorporate adenosine which is rapidly converted into ATP (Su, 1975; Pearson *et al.*, 1978; Pearson & Gordon, 1979). In this way, it seems natural to consider the smooth muscle cells and the endothelial cells of the vascular wall as a likely postjunctional source of ATP.

Because postjunctional release of ATP from vessels can occur in conditions such as hypoxia (Paddle & Burnstock, 1974) it might play an important role in some pathophysiological states. Recently, it has been shown that isolated segments of rabbit aorta submitted to transmural stimulation or exposed to α_1-adrenoceptor agonists released ATP. Endothelial cells were mainly responsible for this release (Sedaa *et al.*, 1990). During shear stress induced by fast flow, endothelial cells from different vascular beds have also been shown to release ATP (Milner *et al.*, 1990a,b) but it is not known if vascular smooth muscle cells express the same property in these experimental conditions.

In the present study, we investigated the release of ATP by smooth muscle cells that were freshly isolated from the rabbit aorta and submitted to increased flow. The results were compared with the release of ATP from endothelial cells isolated from the same animals.

Methods

Cell cultures

Endothelial and smooth muscle cells were obtained from the thoracic aorta of 12-month-old male rabbits (New Zealand strain). Vessels were dissected from heart to diaphragm under sterile conditions. The aortae were placed into cold (4°C) Hanks medium. Connective tissue was removed and the aortae were everted by use of a glass Pasteur pipette with a hook at the end. The everted vessels were incubated for 10 min in M199 medium at 37°C, then pulled onto the end of a 1 ml plastic pipette and incubated for 5 min at 37°C in 0.1% crude collagenase. Endothelial cells were dislodged with a stream of M199 medium supplemented with 10% foetal calf serum and centrifuged (180 *g* for 10 min).

The aorta was cut longitudinally and incubated in a solution of collagenase and elastase (1 mg ml^{-1} and 0.25 mg ml^{-1} respectively) for 40 min. The adventitia was removed and the remaining muscle medial layer was split into rings with forceps. The tissue was then incubated for 2 h in collagenase-elastase (1 mg ml^{-1} and 0.5 mg ml^{-1} respectively). Smooth muscle cells were suspended in M199 supplemented with 10% serum and then centrifuged (180 *g* for 10 min).

After harvesting, endothelial and smooth muscle cells were resuspended in M199 supplemented with 10% foetal calf serum, placed in a 25 mm diameter filter holder containing a 3 μm pore size Millipore filter (MF Millipore membrane, type SS, Millipore, U.S.A.) and allowed to equilibrate for 2 h at 37°C in a 5% CO_2 atmosphere.

Release experiments

Cells were perfused through the Millipore filter at low flow rate (0.5 ml min^{-1}) with oxygenated Krebs buffer at 37°C (composition (mM): NaCl 122, KCl 5.2, $CaCl_2$ 2.4, $MgSO_4$ 1.2, $NaHCO_3$ 25.6, KH_2PO_4 1.2, Na_2EDTA 0.03 and glucose 11) using a variable peristaltic pump (LKB 12000 varioperspex). After a 15 min equilibration, the perfusate was collected every 30 s for 32 min. During the time course of the experiment, cells were stimulated twice by a high flow rate (3.0 ml min^{-1}) for 3 min.

Determination of the number of cells

The protein content of endothelial cells was determined after an overnight incubation of the filter at 4°C in a sterile solution of 0.1% Triton X100 in phosphate-buffered saline according to the method developed by Bradford (1976). The number of cells was estimated by comparing the given value with a standard curve correlating the protein content of the cells with the number of released nuclei obtained after treatment of the

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filters with a hypotonic solution of citric acid containing 0.1% (w/v) crystal violet (Van Wezel, 1973). The number of smooth muscle cells was estimated, prior to the experiment, by counting.

Lactate dehydrogenase (LDH) activity measurements

Endothelial cells from a rabbit aorta were placed on two filters. One filter was processed for perfusion and the other was incubated in oxygenated Krebs solution. After the experiment, the two filters were incubated in 1 ml of a solution of 0.1% Triton X100 for 2 h at 20°C and homogenized; 0.5 ml of the homogenate was then incubated at 37°C in 1 ml of phosphate-buffered saline containing pyruvate (0.75 mM) and NADH (1.28 mM). After 30 min, 1 ml colour reagent was added and the filters were incubated for 20 min at 20°C; 5 ml of 0.4 M NaOH was then added to each sample. Absorbance was read at 442 nm with a spectrophotometer. LDH activity in the Krebs perfusate (0.5 ml samples) was quantified in the same way.

ATP measurements

After the experiment, 100 μl of the perfusate from each fraction was transferred into polypropylene tubes and left at 4°C for 2 h. ATP quantification was performed on a Packard luminometer by addition of 200 μl of luciferase-luciferin (3.33 mg ml^{-1}) to the sample as previously described (Kirkpatrick & Burnstock, 1987).

Drugs

M199 medium and Hanks balanced salt solution were from Gibco, U.K. Foetal calf serum was from Flow Laboratories, U.K. Collagenase (crude) was from Boehringer-Mannheim, West Germany. Elastase (Type III), luciferase-luciferin and the LDH colour reagent were from Sigma, U.S.A.

Statistics

Results are expressed as pmol of ATP released per ml per million cells and are presented as mean values ± s.e.mean. Results were analysed and compared by a Wilcoxon-Mann-Whitney test and interpreted as significantly different at $P < 0.005$. n refers to the number of experiments performed.

Results

Figure 1 shows results on release of ATP from endothelial and smooth muscle cells during periods of increased flow. During low flow rate (0.5 ml min^{-1}), ATP is released from both endothelial (Figure 1a) and smooth muscle cells (Figure 1b). This basal release (first 5 min) is not significantly different ($P = 0.08$) in endothelial cells (0.0335 ± 0.0038 pmol ml^{-1} per 10^6 cells) and in smooth muscle cells (0.0265 ± 0.0033 pmol ml^{-1} per 10^6 cells). During periods of increased flow rate (3.0 ml min^{-1}), ATP release from endothelial cells was rapidly and significantly increased. This release of ATP was significantly greater during the first period of increased flow (0.4662 ± 0.0435 pmol ml^{-1} per 10^6 cells) than in the second one (0.16375 ± 0.0192 pmol ml^{-1} per 10^6 cells). In smooth muscle cells the amounts of ATP released during the first (0.0333 ± 0.0023 pmol ml^{-1} per 10^6 cells) and second periods of stimulation (0.0280 ± 0.0024 pmol ml^{-1} per 10^6 cells) were not significantly different ($P = 0.2$) from the basal release and were not significantly different ($P = 0.1$) from each other.

Figure 2 represents the effects of three consecutive periods of increased flow on the release of ATP by freshly isolated endothelial cells. The basal release of ATP (first 5 min, 0.0278 ± 0.0028 pmol ml^{-1} per 10^6 cells) was significantly increased during these three periods. However, the amount of

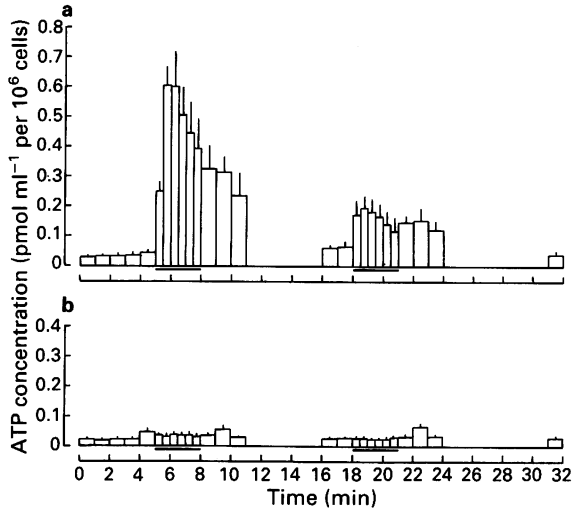

Figure 1 ATP release in (a) endothelial cells and (b) smooth muscle cells freshly isolated from rabbit thoracic aorta. Periods of increased flow (3 ml min^{-1}) are indicated by a horizontal bar. Results are expressed as picomol of ATP released ml^{-1} per 10^6 cells. $n = 4$.

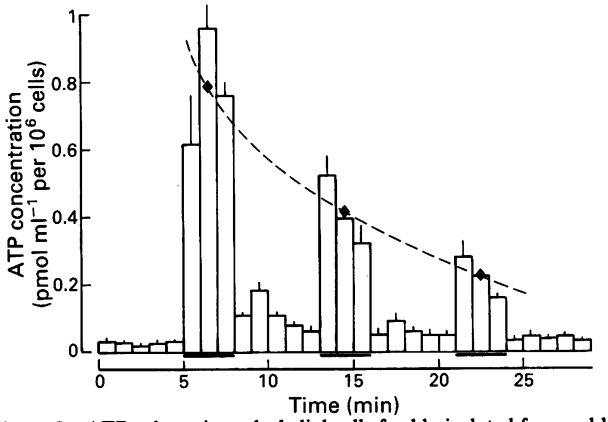

Figure 2 ATP release in endothelial cells freshly isolated from rabbit thoracic aorta. Periods of increased flow (3 ml min^{-1}) shown by a horizontal bar. (◆) Mean of ATP released during stimulation; (– – –) exponential regression. $n = 3$. Results are expressed as picomol of ATP released ml^{-1} per 10^6 cells.

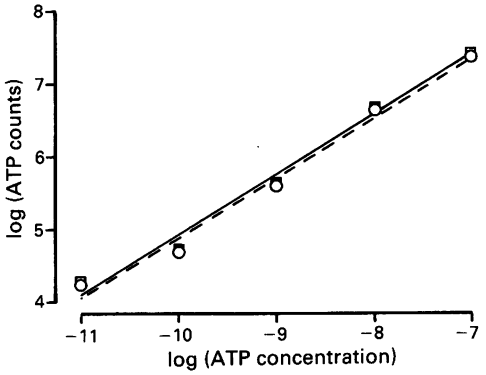

Figure 3 Activity of exonucleases. Endothelial cells were perfused at low flow rate (0.3 ml min^{-1}) for 5 min with Krebs solution containing increasing concentrations of ATP. Continuous line: ATP concentration in Krebs solution before passage over the cells; broken line: ATP concentrations in Krebs solution after passage over the cells. $n = 3$.

ATP released during the first stimulation was greater than that released during the second, which itself was greater than the amount released during the third stimulation. Statistical analysis showed that this decrease was exponential (correlation coefficient = 0.999).

In order to assess the activity of exonucleases, endothelial cells were perfused at low flow rate (0.3 ml min^{-1}) in the presence of different concentrations of ATP in the Krebs buffer. Results, presented on Figure 3, show that ATP concentration

is not significantly different (−2%) in the Krebs buffer collected before perfusion of the cells from that in the buffer collected after passage over the cells.

LDH activity was measured in the perfusate (Krebs buffer) and was not detectable at fast flow or low flow rate. LDH activity measured after treatment of endothelial cells on the filter with Triton X100 was slightly smaller (95.6 ± 1.2%, $n = 4$) in perfused cells than in control cells (100%).

Discussion

In this study we have shown that smooth muscle cells freshly isolated from the rabbit thoracic aorta are unable to release ATP when subjected to increased flow. In contrast, endothelial cells freshly isolated from the same vascular bed of the same animals released ATP when stimulated.

This feature is unlikely to be due to the different enzymatic treatments of the two cell types, given that the same differences have been observed in preliminary experiments on cultured endothelial and smooth muscle cells (data not shown). The release of ATP by freshly isolated endothelial cells during periods of increased flow does not seem to result from cell lysis since release of this agent may be reproduced several times and since the lactate dehydrogenase activity is not detectable in the perfusate and only slightly reduced (−4.4%) after the experiment, in perfused cells. It is also improbable that this release results from non-specific mechanisms since vasopressin, which is present in the cells, is not released during periods of fast flow (Milner *et al.*, 1990a).

There was a larger amount of ATP released by endothelial cells during the first period of increased flow than during the second. In fact, successive stimulations of endothelial cells by fast flow showed that the decrease in the release of ATP was exponential. The main source of ATP is adenosine (Pearson *et al.*, 1978). Since there is no adenosine in the Krebs perfusion medium, the concentration of this substrate may be limiting so that the synthesis of ATP during the experiment is reduced. This may, at least partly, explain this decrease.

ATP is released by nerve varicosities, hypoxic erythrocytes (Bergfeld & Forester, 1989) and aggregating blood platelets. The vascular receptors mediating responses to ATP have been distinguished into two subtypes: P_{2X}-purinoceptors are located on smooth muscle cells and mediate vasoconstriction, P_{2Y}-purinoceptors are present on endothelial cells and mediate vasodilatation (Burnstock & Kennedy, 1985). These opposing P_2-purinoceptor responses can occur in the same tissue, as has been demonstrated, for example, in the aorta (White *et al.*, 1985) and coronary arteries (Houston *et al.*, 1987). In this way, ATP released by nerves is able to generate a vasoconstriction acting directly on smooth muscle cells via P_{2X}-purinoceptors. On the other hand, ATP released by platelets or by erythrocytes provokes the relaxation of the vessel via the release of endothelium-derived relaxing factor (EDRF) acting on P_{2Y}-purinoceptors on endothelial cells.

An increase in blood flow provokes a vasodilatation and this is not due to passive distension of the vessel wall (Hull *et al.*, 1986). After removal of the endothelium, vessels do not dilate to increased flow. It has already been shown that this mechanical stimulation acts by releasing EDRF from the endothelium (Hull *et al.*, 1986). Our results indicate that during periods of fast flow. ATP is released by endothelial cells but not by smooth muscle cells. This suggests that, of these two major cell types in the vascular wall, it is only endothelial cells which react to shear stress by releasing ATP.

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