0014-4754/95/030256-04$1.50 + 0.20

Synergistic effect of acute hypoxia on flow-induced release of ATP from cultured endothelial cells

P. Bodin and G. Burnstock*

Department of Anatomy and Developmental Biology, University College London, Gower Street, London, WC1E 6BT (England, UK)
Received 22 August 1994; received after revision 25 October 1994; accepted 31 October 1994

Abstract. Human umbilical vein endothelial cells (HUVECs) in primary cultures were perfused under normoxic or hypoxic conditions. These cells were stimulated twice for 3 min by increased flow (from 0.5 to 3.0 ml/min). Under hypoxic conditions the basal release of ATP was the same as under normoxic conditions, but during increased flow the release was greater ($0.58 \pm 0.07 > 0.32 \pm 0.04$ pmoles/ml/10^6 cells (+78%), for the first period of stimulation; $0.39 \pm 0.05 > 0.22 \pm 0.03$ pmoles/ml/10^6 cells (+79%) for the second period). Further experiments with sequential increments in flow rate showed that under both normoxic and hypoxic conditions, a positive correlation existed between ATP release and the rate of flow but there was always more ATP released under hypoxic conditions regardless of the flow rate.

HUVECs in secondary culture (second passage) were similarly stimulated. No differences were observed between normoxic and hypoxic conditions. In both cases, the quantity of ATP released during high flow (0.050 ± 0.004 pmoles/ml/10^6 cells) was significantly smaller than the quantity of ATP released during low flow (0.09 ± 0.01 pmoles/ml/10^6 cells).

To conclude, since hypoxia alone did not affect ATP release, there appears to be a synergistic relationship between increased shear stress and hypoxia in the stimulation of ATP release from HUVECs. Moreover, the release of ATP under these conditions seems to be a property of highly differentiated endothelial cells.
Key words. Vascular endothelial cells; cultures; ATP; acute hypoxia; perfusion; increased flow.

Adenosine-5′-triphosphate (ATP) is a potent mediator in the local control of the vascular tone[1]. As a neurotransmitter, ATP is co-released with noradrenaline upon sympathetic nerve stimulation and acts on P_{2x}-purinoceptors located on vascular smooth muscle cells, mediating vasoconstriction of most vessels[2].

ATP is also released into the lumen of vascular beds such as the mesenteric[3], cerebral[4], pulmonary[5] and coronary bed[6] during experimental conditions of increased flow or hypoxia. ATP can then act on P_{2Y}-purinoceptors present on vascular endothelial cells to mediate dilatation[7,8] via the release of nitric oxide[9].

Amongst the different vascular cell types, it is probable that endothelial cells are the main source of this extraneuronal ATP[10,11]. Using either freshly isolated endothelial cells or cells in primary culture, release of ATP has already been demonstrated during increased shear stress[11,12]. ATP is released together with other vasoactive substances, including endothelin or substance P[6,13–15]. The amount of ATP released during increased shear stress has already been shown to be affected by the age of the animals[16] and is reduced after animals have been exposed to conditions of chronic hypoxia[17].

Acute hypoxia often results in changes in blood flow[18]. In this paper, the effect of hypoxia on the release of ATP by increased shear stress has been investigated on endothelial cells from human umbilical vein in culture.

Materials and methods

Isolation of endothelial cells. Umbilical cords were provided by the Labour Ward of University College Hospital, London. Cords obtained after normal deliveries were placed in cold (4 °C), sterile Hepes Buffered Saline (HBS; composition mM: NaCl 137, KCl 4, glucose 11, Hepes HCl 10, pH 7.4 at 37 °C)[19]. Each cord (20 cm piece) was wiped clean with a sterile gauze, cannulated at both ends and flushed with 60 ml of HBS at 37 °C. The vein was filled with a solution of 0.1% collagenase (type II, Worthington, NJ, USA) in HBS and incubated at 37 °C for 10 min in a beaker filled with sterile warm HBS. After the incubation period, the enzyme solution was flushed out of the vein with 50 ml of HBS at 37 °C. The cell suspension was centrifuged for 10 min at 180 × *g*. Endothelial cells were resuspended in culture medium: M199 (Gibco, UK) supplemented with 20 mM Hepes and 15% foetal calf serum (Flow laboratories, UK). Cells from one umbilical vein were plated on 2 sterile cellulose membranes (Millipore, type SS, ϕ

* To whom correspondence should be addressed.

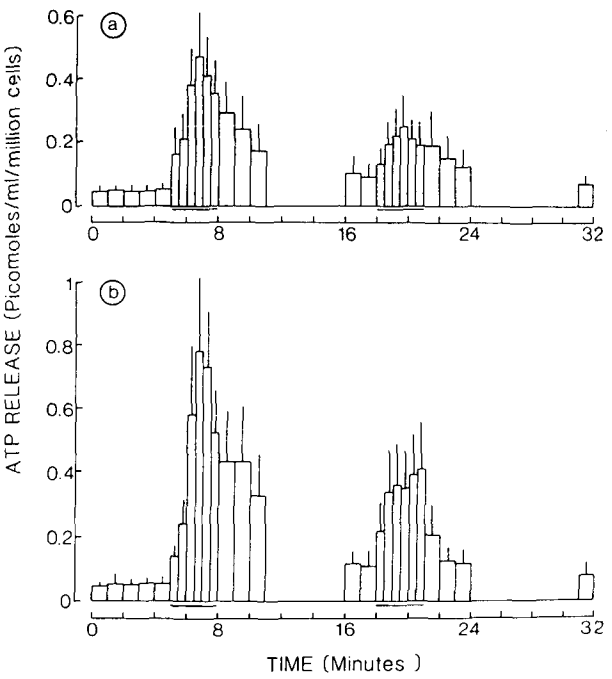

Figure 1. *a* Endothelial cells in primary culture perfused with normoxic krebs at 0.5 ml/min and stimulated twice for 3 min by increased flow (3.0 ml/min, ——).
b Endothelial cells perfused at 0.5 ml/min with hypoxic krebs and stimulated twice for 3 min by increased flow (3.0 ml/min, ——). Each bar represents the mean ± SEM of 5 experiments.

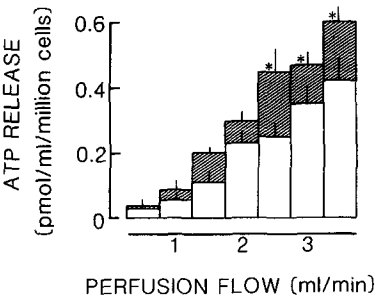

Figure 2. Release of ATP by HUVECs in primary culture under normoxic conditions (□), and under hypoxic conditions (▨), n = 3. Endothelial cells were perfused for 3 min at each flow rate (0.5; 1; 1.5; 2; 2.5; 3 and 3.5 ml/min). The perfusate was collected only during the last 30 s of each flow rate to allow for the maximum release of ATP to occur and was analysed for its ATP content. *$p < 0.05$. Each bar represents the mean ± SEM of 3 experiments.

25 mm, 3 µm pore size)[10]. The culture medium was changed after 24 h. The cells were used after 4 days in culture. HUVECs were passaged after treatment with trypsin (0.25%) and replated 1:3 either on plastic Petri dishes (first passage) or on sterile cellulose membranes (second passage).

Release experiments. Release experiments have already been described in detail[11,17]. Briefly, the filter was placed in a plastic filter holder, with care taken not to expose the cells to the atmosphere. Endothelial cells were perfused using a peristaltic pump (Minipump, Verder, Germany) at a basal rate of 0.5 ml/min with 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, glucose 11). This buffer was bubbled with a 95% O_2, 5% CO_2 gas mixture (normoxic conditions) or with a 95% N_2, 5% CO_2 gas mixture (hypoxic conditions) and equilibrated for 1 h before perfusion of the cells. The cells were perfused for 30 min at 0.5 ml/min for equilibration in normoxic or hypoxic Krebs. During the time course of the experiment, cells were stimulated twice by a high flow rate (3.0 ml/min) for 3 min. The perfusate was collected as 1 min samples during low flow rate periods and as 30 s samples during periods of stimulation. In order to study the release from endothelial cells subjected to progressively increased flow, cells were perfused for 3 min at different flow rates ranging from 0.5 to 3.5 ml/min with 0.5 ml/min increments. The perfusate was collected for the last 30 s of each period.

ATP measurements. After the experiment, 100 µl of the perfusate from each fraction was transferred into propylene tubes. ATP quantification was performed on a Packard luminometer by addition of 200 µl of a solution of luciferin-luciferase (3.33 mg · ml^{-1}, Sigma, UK) to the sample as previously described[20]. The number of cells was estimated by determining the protein content of the preparations[11,21].

Statistics. Results are expressed as picomoles of ATP released per ml per million cells and are presented as mean values ± standard error of the mean (SEM). These results were analysed and compared by analyses of variance and interpreted as significantly different at $p < 0.05$. n refers to the number of experiments performed.

Results

Release of ATP by increased flow rate in normoxic conditions. The quantity of ATP released during basal flow (0.053 ± 0.007 picomoles/ml/10^6 cells) was significantly increased during the first (0.327 ± 0.037 picomoles/ml/10^6 cells; $p < 0.001$) and second (0.216 ± 0.03 picomoles/ml/10^6 cells; $p < 0.001$) period of stimulation (fig. 1a). However, the quantity of ATP released during the second period of stimulation was significantly smaller ($p = 0.02$) than that released during the first period.

Release of ATP by increased flow rate in hypoxic conditions. ATP was released when the cells were perfused at low flow rate (0.059 ± 0.009 picomoles/ml/10^6 cells). This release was significantly increased during the first (0.583 ± 0.069 picomoles/ml/10^6 cells; $p < 0.001$) and second (0.387 ± 0.047 picomoles/ml/10^6 cells; $p < 0.001$) period of stimulation (fig. 1b). Furthermore, the quantity of ATP released during these two periods was not significantly different ($p = 0.06$).

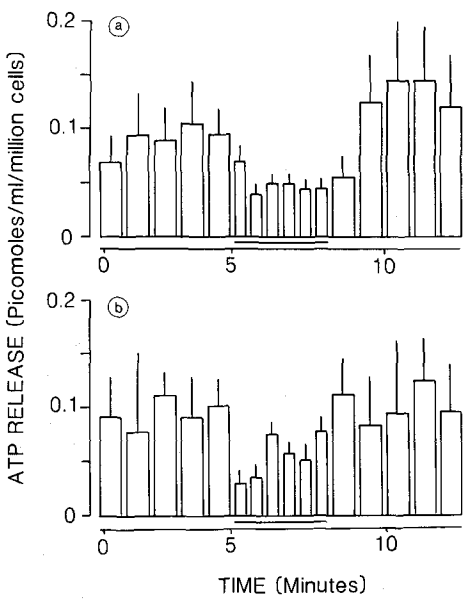

Figure 3. HUVECs in secondary culture (second passage) perfused at 0.5 ml/min and stimulated for 3 min by increased flow (3.0 ml/min).
a Under normoxic conditions, during the period of increased flow (——), the concentration of ATP in the perfusate was significantly reduced ($p < 0.05$).
b Under hypoxic conditions, the quantity of ATP released during perfusion at high flow (——) was also significantly reduced ($p < 0.05$).
No significant differences were observed between the conditions of normoxia and hypoxia. Each bar represents the mean ± SEM of 5 experiments.

The comparison of the experimental conditions of normoxia and hypoxia shows that the quantity of ATP released during the initial period of perfusion (first 5 min at 0.5 ml/min) was not significantly different. Thus, acute hypoxia alone had no effect on ATP release. The amount of ATP release by increased flow under hypoxic conditions was greater than that released in the same conditions under normoxic conditions, for the first (+78%; $p < 0.001$) and second (+79%; $p < 0.001$) period of stimulation.

Release of ATP during step increases of flow. Endothelial cells were subjected to progressive rates of perfusion flow. As shown in figure 2, under normoxic conditions, the quantity of ATP released by endothelial cells was proportional to the perfusion flow rate. Under hypoxic conditions, this release also remained proportional to the perfusion flow rate but the quantity of ATP released was increased and significantly different from 2.5 to 3.5 ml/min when compared to the results obtained under normoxic conditions.

Release of ATP by HUVECs in secondary cultures. As shown in figure 3, human endothelial cells in secondary culture (after only two passages) released ATP when perfused at low flow (0.09 ± 0.01 picomoles/ml/10^6 cells) and this basal release was not significantly different than that of cells in primary cultures. However, HUVECs in secondary cultures did not release more ATP when perfused at a high flow rate. On the contrary, the concentration of ATP in the perfusate released during high flow (0.05 ± 0.004 picomoles/ml/10^6 cells) was significantly lower than during basal low flow ($p < 0.05$). Under hypoxic conditions, no differences in the release of ATP were observed either at low flow (0.09 ± 0.01 picomoles/ml/10^6 cells) or at high flow (0.06 ± 0.01 picomoles/ml/10^6 cells).

Discussion

ATP released during perfusion of freshly isolated or cultured endothelial cells stimulated by increased flow under normoxic conditions has already been clearly demonstrated in cells isolated from rabbit aorta[11,12], rat aorta[17] and human umbilical vein[22]. In this study, we used endothelial cells derived from the human umbilical vein in order to study the effect of acute hypoxia on ATP released by increased flow.

Our results showed acute hypoxia alone did not stimulate ATP release but in conjunction with increased flow, stimulated more release than with increased flow alone. Under normoxic or acute hypoxic conditions the release of ATP by HUVECs was proportional to the flow of perfusion and specific of highly differentiated cells.

Release of ATP from freshly isolated or cultured vascular endothelial cells perfused on filter has already been described[11,17]. During periods of increased flow, endothelial cells concomitantly release endothelin and ATP[12]. This release has been shown to be selective and does not result from lysis of the cells[11]. In the present study, we demonstrate that the release of ATP is a property characteristic of highly differentiated vascular endothelial cells by showing that HUVECs in secondary cultures (after only 2 passages) are no longer able to release ATP when stimulated by increased flow under either normoxic or hypoxic conditions.

ATP release can be stimulated several consecutive times by increased flow, although under these experimental conditions the release decreases exponentially. We previously concluded that this was probably due to a depletion of intracellular ATP stores[11]. In this study, endothelial cells under hypoxic conditions released more ATP during the second stimulation than cells under normoxic conditions, during the first period of stimulation. This last result suggests that the decrease in the release of ATP during consecutive periods of increased flow is probably due to an adaptative mechanism.

The quantity of ATP released under both normoxic and hypoxic conditions increased with increments in perfusion flow rate. Moreover, the quantity of ATP release was greater when the cells were perfused under hypoxic conditions. There are several possibilities for this: first, different mechanisms of release of ATP during periods

of increased flow, under hypoxia or normoxia, may exist which both depend on conditions of shear stress; second, the mechanisms involved in the release of ATP during stimulation of the cells by increased flow may be the same under normoxic and hypoxic conditions but the mechanism may be amplified under conditions of hypoxia. Alternatively, during increased flow, endothelial cells may release other vasoactive substances such as nitric oxide, prostacylins, endothelin, serotonin and substance P[6,23-26] which may be responsible for the release of ATP during periods of increased flow under hypoxic conditions. However, unless this mechanism only operates under conditions of shear stress, it is unlikely that one of these agents is involved given that the quantities of ATP released are not significantly different when the cells are perfused at a low flow rate under normoxic or hypoxic conditions.

The release of ATP during hypoxia has already been demonstrated in the Langendorf heart preparation[18,27]. If one assumes that the mechanism of ATP release during hypoxia is not different in endothelial cells isolated from umbilical vein or from the intact coronary bed, it is possible that ATP is released in response to changes in flow induced by hypoxia and then reinforces the hypoxic stimulus itself. Alternatively, the mechanism of ATP release during hypoxia could vary according to vascular beds. Results obtained using HUVECs have also been obtained with endothelial cells isolated from rabbit aorta (data not shown). However, these two vascular beds are known to have little or no sympathetic innervation and are elastic vessels in contrast to the vessels of the coronary circulation.

We have already shown that when rats are exposed to chronic hypoxia, ATP release from freshly isolated endothelial cells from the aorta is decreased during stimulation of increased flow[17]. The differences between the effects of long-term hypoxia in vivo and acute hypoxia in vitro may reflect an adaptative mechanism of endothelial cells exposed to reduced arterial oxygen tension for a long period of time[17].

In summary, ATP is released by endothelial cells from human umbilical vein in primary cultures and this release is proportional to the shear stress imposed on the cells. Actute hypoxia alone does not induce ATP release but there is a synergistic relationship between acute hypoxia and increased shear stress; under hypoxic conditions, more ATP is released in response to increased flow. The release properties of HUVECs are lost once the cells are replated in secondary cultures and thus appear to be a property characteristic of highly differentiated endothelial cells. It is possible that in vivo, vascular endothelial cells continuously release ATP as a regulator of vascular tone[4]. This release is increased in situations where hypoxia and/or shear stress is imposed on the cells, thus resulting in dilatation of the vascular bed.

Acknowledgments. This work was supported by the British Heart Foundation. The authors wish to thank the nursing staff of the Obstetric Department of University College Hospital for collecting the umbilical cords.

1 Burnstock, G., Ann. N.Y. Acad. Sci. *603* (1990) 31.
2 Burnstock, G., Trend. Pharmac. Sci. *9* (1988) 116.
3 Ralevic, V., Milner, P., Kirkpatrick, K. A., and Burnstock, G., Experientia *48* (1992) 31.
4 Domer, F. R., Alexander, B., Milner, P., Bodin, P., and Burnstock, G., Brain Res. *630* (1993) 88.
5 Hassèssian, H., Bodin, P., and Burnstock, G., Br. J. Pharmac. *109* (1993) 466.
6 Milner, P., Ralevic, V., Hopwood, A. M., Fehér, E., Lincoln, J., Kirkpatrick, K., and Burnstock, G., Experientia *45* (1989) 121.
7 Burnstock, G., and Kennedy, C., Gen. Pharmac. *16* (1985) 433.
8 Ralevic, V., and Burnstock, G., Neural-endothelial Interactions in the Control of Local Vascular Tone. Medical Intelligence Unit. RG Landes Company, Medical Publishers, Austin Texas, 1993.
9 Hopwood, A. M., Lincoln, J., Kirkpatrick, K. A., and Burnstock, G., Eur. J. Pharmac. *165* (1989) 323.
10 Sedaa, K. O., Bjur, R. A., Shinozvka, K., and Westfall, D. P., J. Pharmac. exp. Ther. *252* (1990) 1060.
11 Bodin, P., Bailey, D., and Burnstock, G. Br. J. Pharmac. *103* (1991) 1203.
12 Milner, P., Loesch, A., and Burnstock, G., Biochem. biophys. Res. Commun. *170* (1990) 649.
13 Rubanyi, G. M., and Vanhoutte, P. M., J. Physiol. *364* (1985) 45.
14 Rubanyi, G. M., Romero, J. C., and Vanhoutte, P. M., Am. J. Physiol. *250* (1986) H1145.
15 Rakugi, H., Yoshikatsu, T., Nakamaru, M., Nagano, M., Highashimori, K., Mikami, H., Ogihara, T., and Suzuki, N., Biochem. biophys. Res. Commun. *169* (1990) 973.
16 Milner, P., Bodin, P., Loesch, A., and Burnstock, G., J. vasc. Res. *29* (1992) 420.
17 Bodin, P., Milner, P., Winter, R., and Burnstock, G., Proc. R. Soc. B. *247* (1992) 131.
18 Paddle, B. M., and Burnstock, G., Blood Vessels *11* (1974) 110.
19 Jaffe, E. A., Nachman, R. L., Becker, C. G., and Minick, C. R., J. clin. Invest. *52* (1973) 2745.
20 Kirkpatrick, K., and Burnstock, G., Eur. J. Pharmac. *138* (1987) 207.
21 Bradford, M. M., Analyt. Biochem. *72* (1976) 248.
22 Milner, P., Kirkpatrick, K. A., Ralevic, V., Toothill, V., Pearson, J., and Burnstock, G., Proc. R. Soc. B. *241* (1990) 245.
23 Furchgott, R. F., and Zawadzki, J. V., Nature *288* (1980) 375.
24 Palmer, M. J., Ferige, A. G., and Moncada, S., Nature *327* (1987) 524.
25 Moncada, S., Palmer, R. M. J., and Higgs, E. A., in: Thrombosis and Haemostasis, p. 597. Eds J. Verstraete, J. Vermylen and H. H. R. Lijnen. Leuven University Press, Leuven 1987.
26 Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki. Y., Goto K., and Masaki, T., Nature *332* (1988) 411.
27 Clemens, M. G., and Forester, T., J. Physiol. *312* (1980) 143.