Effects of Adenine Nucleotides on the Proliferation of Aortic Endothelial Cells

P. Van Daele, A. Van Coevorden, P.P. Roger, and J.-M. Boeynaems

The effects of adenine nucleotides and adenosine on DNA synthesis and cell growth have been studied in bovine aortic endothelial cells (BAECs). ATP produced a small but significant (+44%) increase of the fraction of BAECs whose nuclei are labeled by [3H]thymidine. This mitogenic effect was mimicked by ADP, the phosphorothioate analogues ATPγS and ADPβS, and the nonhydrolyzable analogue adenosine 5’-(β,γ-imido)triphosphate (APPNP), whereas adenosine 5’-(α,β-methylene)triphosphate (APCPP), a selective agonist of P2x-purinoceptors, had no effect at 10 μM and a small one at 100 μM; this profile is consistent with the involvement of P2x-receptors. Adenosine induced a mitogenic response of a magnitude similar to that of ATP. This effect was not reproduced by R-phenylisopropyl adenosine, by 5’-N-ethylcarboxamide adenosine, or by 2’,5’-dideoxyadenosine, selective ligands of the A1- and A2-receptors and the P site, respectively, nor was it inhibited by 8-phenyltheophylline, an antagonist of both A1- and A2-receptors. The mechanism of this adenosine action thus remains unclear. ATP and ATPγS did not enhance the proliferation of BAECs cultured in the presence of fetal calf serum concentrations ranging from 0.5% to 10%. They inhibited the growth-promoting effect of basic fibroblast growth factor; among the various nucleotides tested, APCPP was the least effective to reproduce the action of ATP, suggesting the possible involvement of P2x-receptors. In conclusion, the action of ATP on the proliferation of BAECs is complex: an increase in the fraction of cells synthesizing DNA, no effect on the cell proliferation in the presence of serum, and inhibition of the growth-promoting effect of basic fibroblast growth factor. (Circulation Research 1992;70:82–90)

ATP induces a rapid release of endothelium-derived relaxing factor (nitric oxide) and prostacyclin (prostaglandin I2) from vascular endothelial cells. These actions, which are mediated by P2x-receptors, are obtained in a range of ATP concentrations (1–100 μM) comparable to those obtained in plasma after platelet degranulation. They might thus play an important role in the interaction between platelets and the endothelium. In endothelial cells, as in other cells, P2x-receptors are coupled to a phospholipase C that hydrolyzes phosphatidyl-inositol bisphosphate. As a consequence of this initial event, ATP induces in aortic endothelial cells several biochemical responses that are often associated with a mitogenic action: enhanced phosphorylation of 27-kd proteins, stimulation of phosphati-
dylcholine hydrolysis by a phospholipase D, cytoplasmic alkalization via the activation of the Na+/H+ antiport, increased expression of c-fos and c-myc proto-oncogenes. Therefore, we have investigated the effects of ATP on the proliferation of bovine aortic endothelial cells (BAECs).

Materials and Methods

Cell Culture

BAECs were obtained by collagenase digestion of a bovine aorta, as previously described. The cells were seeded on 100-mm Petri dishes and incubated at 37°C in a 5% CO2-humidified incubator in the following medium: minimum essential medium-d-valine (MEM-d-Val) (80% [vol/vol]), fetal calf serum (FCS, 20% [vol/vol]), 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml amphotericin B. MEM-d-Val was used to prevent the proliferation of any contaminating smooth muscle cells. The medium was changed the following day and renewed twice a week. After 4 or 5 days, the primary cultures formed confluent monolayers and could be subcultured. The cells were detached by a 5-minute incubation in a Ca2+- and Mg2+-free Hanks’ buffer containing trypsin (1 mg/ml) and EDTA (1 mM). Thereafter, the

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cells were maintained and subcultured in the following medium: Dulbecco’s modified Eagle’s medium (DMEM, 60% [vol/vol]), Ham’s F12 (20% [vol/vol]), and FCS (20% [vol/vol]), with the same concentrations of penicillin, streptomycin, and amphotericin B as mentioned above.

Mitogenic Assay

For DNA synthesis measurements, BAECs were used after three or four passages. In general, cells were plated at an initial density of $4 \times 10^4$ cells per 35-mm dish and cultured in the same medium as described, but with a reduced concentration of FCS (2.5% [vol/vol]). The cells were allowed 4 hours to attach. Then, the medium was removed, and the culture was pursued in the same medium as described, except for the complete lack of FCS. After 24 hours, the tested agents were added; 20 hours later, the medium was replaced by fresh medium containing $[^3]H$thymidine (1 $\mu$M, 1 $\mu$Ci/ml). This incubation was started after 2 hours. DNA synthesis was estimated by the incorporation of $[^3]H$thymidine into 10% trichloroacetic acid (TCA)-precipitable material or by the frequency of $[^3]H$thymidine–labeled nuclei detected by autoradiography. For the measurement of $[^3]H$thymidine incorporation, the experiments were stopped by rapid removal of the medium, followed by addition of distilled water (1 ml). The cells were scraped, and 100 $\mu$l of a 1 g/dI solution of bovine serum albumin, used as carrier, was added; cold TCA was then added to a final concentration of 5%. After a few hours at 4°C, the samples were filtered under vacuum. After rinsing with 5% cold TCA, the samples on glass fiber filter were solubilized in Soluene and counted in liquid scintillation. For autoradiography, the cells were fixed in methanol and then extensively washed. Autoradiography was performed directly in the Petri dishes, as described by Roger et al. The cells were stained with toluidine blue (0.2%), and the proportion of labeled nuclei was evaluated by counting at least 1,000 nuclei from different microscopic fields. Silver grains were restricted to the nuclei.

Results are expressed as mean±SD of measurements in quadruplicate Petri dishes. All results presented were confirmed with at least two or three independent cultures.

Statistical analysis of the data was performed using the analysis of variance.

Proliferation Assay

BAECs were seeded at an approximate density of 200,000 cells per 35-mm dish in the usual culture medium, containing 2.5% FCS. After 4 hours, the medium was replaced by fresh medium without serum. The experiment was started 24 hours later; the culture media containing a variable proportion of FCS and the tested agents were added at that time and renewed every other day. At the indicated times, the medium was removed, and the cells were washed twice with DMEM and incubated for 2 minutes in 0.5 ml Hanks’ buffer containing trypsin (1 mg/ml). Thereafter, 0.5 ml complete culture medium containing 20% FCS was added to stop trypsin action, and the cells were counted in a hemocytometer. For each dish, the result is the mean of four separate counts; for each condition, results are expressed as the mean±SD of triplicate dishes.

Cytotoxicity Assay

BAECs were cultured for 24 hours in the presence of $[^3]H$thymidine (5 $\mu$Ci/ml). After trypsin treatment, they were seeded at a density of 40,000 cells per 35-mm dish, in the presence of 2.5% FCS. After 4 hours, the medium was replaced by fresh medium with no serum. The medium was renewed again 24 hours later, and the cells were then incubated for 24 hours with the tested agents. Aliquots of the incubation fluid were removed at various time for liquid scintillation counting of the released radioactivity. The residual cell-associated radioactivity was counted after lysis in a buffer containing 0.1% sodium dodecyl sulfate. The ratio between released and cell-associated radioactivities constitutes an index of cytotoxicity.

Materials

Collagenase type IA was purchased from Cooper Biomedical (Worthington Biochemical Corp., Freehold, N.J.). DMEM, Ham’s F12, MEM-d-Val, FCS, penicillin, streptomycin, amphotericin B, and glucose were from Gibco Laboratories, Grand Island, N.Y. Trypsin was obtained from Flow Laboratories, Inc. [Methyl-2$^3$H]thymidine (40 Ci/mmole) was from Amersham Corp. ATP, ADP, adenosine, hypoxanthine, inosine, adenosine 5’-(a,β-methylene)triphosphate (ACPP), adenosine 5’-(β,γ-imido)triphosphate (APPNP), ITP, UTP, R-phenylisopropyl adenosine (R-PIA), 5’-N-ethylcarboxamide adenosine (NECA), bradykinin, and cycloheximide were obtained from Sigma Chemical Co., St. Louis, Mo. Adenosine 5’-O-(3-thio)triphosphate (ATP$\gamma$S), adenosine 5’-O-(2-thio)diphosphate (ADP$\beta$S), and GTP were purchased from Boehringer Mannheim Corp. 8-Phenythiofylline was obtained from Research Biochemicals Inc., and 2’,5’-dideoxyadenosine (DDA) was purchased from Pili Biochemicals. Human recombinant basic fibroblast growth factor (bFGF) was obtained from Amersham or Boehringer Mannheim. Human recombinant tumor necrosis factor (TNF) was a gift of Dr. W. Fiers.

Results

In preliminary experiments, changes in endothelial cell proliferation rate were evaluated by the measurement of $[^3]H$thymidine incorporation into TCA-insoluble material. ATP increased this incorporation in a concentration-dependent manner (Figure 1). The minimally effective concentration of ATP (1 $\mu$M) produced a significant increase of $[^3]H$thymidine incorporation above the control: 76% (mean of two experiments, range, 68–84%). The maximal effect of ATP was obtained at a 100-μM concentration, which
induced a threefold increase of [3H]thymidine incorporation, whereas this incorporation was stimulated approximately eightfold by 10% FCS. The effect of ATP (100 μM) was not influenced by the initial seeding density, from 0.4×10³ to 1.5×10³ cells (Table 1).

In further experiments, the mitogenic action of the agents tested was evaluated more directly by the proportion of labeled nuclei after incubation with [3H]thymidine. Despite a 24-hour withdrawal from serum, the cells were not quiescent as previously observed by other authors using a similar protocol. Under control conditions, 29±8 nuclei out of 100 were labeled (mean±SD, 22 experiments, range, 17–36). ATP (10–100 μM) increased this labeling index to 42±6% (mean±SD, 19 experiments, range, 34–57%), which represents a 44% increase (Figure 2). This stimulation was highly significant (p<0.01). ADP mimicked the effect of ATP; at 10 μM, it raised the labeling index to 165±13% of the control value (mean±SD, five experiments, range, 133–195%, p<0.01) (Figure 2). The effect of ATP and ADP was reproduced by their phosphorothioate analogues, ATP[S] and ADP[S] (data not shown). The effect of ATP on thymidine incorporation was also mimicked by the nonhydrolyzable analogue APPNP, which at 100 μM was equiactive to ATP (Figure 3). On the contrary, APCPP, a selective agonist of P2Y-receptors, was inactive at 10 μM and produced a smaller effect than ATP itself at 100 μM (Figure 3). ITP and GTP, but not UTP, were also active (Figure 2). The effect of ATP was not mimicked by bradykinin, which is

**TABLE 1. Effect of Seeding Density on [3H]Thymidine Uptake by Cultured Bovine Aortic Endothelial Cells**

<table>
<thead>
<tr>
<th>Cell density (×10³/dish)</th>
<th>Control</th>
<th>100 μM ATP</th>
<th>10% FCS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm×10³ per dish</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.4</td>
<td>4.6±1.0</td>
<td>9.8±0.5</td>
<td>26.4±4.3</td>
</tr>
<tr>
<td>0.5</td>
<td>5.4±1.6</td>
<td>15.0±4.9</td>
<td>33.5±0.7</td>
</tr>
<tr>
<td>1</td>
<td>15.0±5.6</td>
<td>32.0±6.7</td>
<td>61.0±7.2</td>
</tr>
<tr>
<td>1.5</td>
<td>27.7±1.0</td>
<td>54.0±5.8</td>
<td>85.0±10.8</td>
</tr>
</tbody>
</table>

Values are mean±SD and represent the incorporation of [3H]thymidine radioactivity into trichloroacetic acid-insoluble material. FCS, fetal calf serum. They are expressed as the mean±range of measurements in duplicate dishes in one representative experiment out of two.
known to induce the same repertoire of biochemical events as ATP in BAECs \(^1-^3,^6,^7,^9\) (Figure 4).

Adenosine also increased the rate of thymidine incorporation in endothelial cell cultures with an amplitude equivalent to that of ATP (Figure 2). It increased the labeling index to 40\(\pm\)9% (mean\(\pm\)SD, nine experiments, range, 33\(-\)46%). This stimulation was highly significant \((p<0.01)\). The effect of adenosine was mimicked by its metabolites inosine and hypoxanthine (Figure 5). On the contrary R-PIA and NECA, selective agonists for A\(_1\) and A\(_2\)-receptors, respectively, were completely inactive (Figure 6). To determine whether the effect of adenosine was mediated by membrane receptors of the P\(_1\) type, the effect of 8-phenyltheophylline was tested. This antagonist did not significantly decrease the mitogenic effect of adenosine (Figure 6). On the other hand, the effect of adenosine was not reproduced by the P-site ligand DDA (Figure 7).

At confluence, the labeling index of BAECs was reduced as compared with that of sparse cells: a significant increase of this index could no longer be detected in response to ATP, adenosine, or serum (Figure 8). This result is consistent with the concept that endothelial cell growth is subject to a true cell-contact inhibition. After a 24-hour exposure to ATP or adenosine, comparable to that used in the mitogenic assay, there was no evidence of cytotoxicity, as measured by the release of radioactivity from \([\text{H}]\)thymidine–labeled BAECs (Figure 9). A positive control was provided by TNF, which, as expected, induced lysis of BAECs.\(^{13}\)

In none of 14 experiments did ATP or ATP\(_x\) stimulate the proliferation of BAECs (Figures 10 and 11). The same negative results were obtained whatever the concentration of FCS: 0.5% or 10\% (Figure 10), 1\% (Figure 11), and 2.5\% or 5\% (data not shown). Furthermore, ATP slowed down the proliferation of BAECs induced by bFGF (Figure 11). This growth-inhibitory action of ATP was fully mimicked.

**FIGURE 4.** Bar graph comparing the effects of ATP and bradykinin on DNA synthesis in bovine aortic endothelial cells. After a 24-hour serum deprivation, the cells were incubated for 20 hours in the presence of ATP, bradykinin (Brady), or fetal calf serum (FCS) and then for 2 hours with \([\text{H}]\)thymidine. Results are expressed as the percent of \([\text{H}]\)thymidine–labeled nuclei. They represent the mean\(\pm\)SD of measurements in triplicate dishes in one representative experiment out of two. Values are significant at \(p<0.01\) for the effects of ATP and FCS vs. control (Cont).

**FIGURE 5.** Bar graph showing stimulation of DNA synthesis in bovine aortic endothelial cells by adenosine: comparison with hypoxanthine and inosine. FCS, fetal calf serum. After a 24-hour period of serum deprivation, the cells were incubated for 20 hours with the various agents tested and then for 2 hours with \([\text{H}]\)thymidine. Results are expressed as the fraction of cells whose nuclei are labeled by \([\text{H}]\)thymidine. They represent the mean\(\pm\)SD of measurements in quadruplicate dishes in one representative experiment out of two. Values are significant at \(p<0.01\) for adenosine, hypoxanthine, and inosine vs. control.

**FIGURE 6.** Bar graph showing effects of agonists and an antagonist of adenosine (ADO) receptors on DNA synthesis in bovine aortic endothelial cells. FCS, fetal calf serum; PIA, phenylisopropyl adenosine; NECA, 5'-N-ethylcarboxamide adenosine; 8-PT, 8-phenyltheophylline. After a 24-hour period of serum deprivation, the cells were exposed for 20 hours to the various agents and then incubated for 2 hours with \([\text{H}]\)thymidine. Results are expressed as the percent of \([\text{H}]\)thymidine–labeled nuclei. They represent the mean\(\pm\)SD of measurements in quadruplicate dishes in one experiment out of two. Values are significant at \(p<0.01\) for adenosine and 8-PT+ADO vs. control.
by ATPγS, ADP, ADPβS, and APPNP, but only partially by APCPP (Table 2).

Discussion

ATP produced a slight but significant increase in the fraction of BAECs that incorporate [3H]thymidine in their nuclei, indicating a stimulation of DNA synthesis. However, ATP and various analogues did not stimulate the proliferation of BAECs per se and did not potentiate the effect of suboptimal serum concentrations. Furthermore, ATP inhibited the growth-promoting effect of bFGF. The apparent discrepancy between an increased rate of DNA synthesis and no increase in cell density is reminiscent of in vivo observations reported in the literature. Endotoxin injection increases cell replication in the rat aortic endothelium without any change in cell density; presumably endotoxin produces desquamation with simultaneous replacement by cell replication.15 In uninjured rat arteries, systemic bFGF also increases the replication rate of endothelial cells but has no effect on cell density.16 It might be that ATP triggers the apoptosis of aortic endothelial cells, as it does with other cells,17 although no increase in the extracellular release of radioactivity from [3H]thymidine–labeled BAECs could be detected over a 24-hour period. Whether there is any relation between the effects of ATP and adenosine on the labeling index of BAECs, as described here, and the angiogenic activity of these compounds, observed in vivo,18,19 remains hypothetical.
In terms of concentration–action and agonist specificity, the mitogenic effect of ATP, described in this study, is quite similar to other actions of ATP mediated by P2Y-receptors.\(^1\)\(^-\)\(^3\)\(^-\)\(^5\)\(^-\)\(^7\) However, bradykinin, whose receptors are coupled to phospholipase C just like P2Y-receptors and which induces the same “early mitogenic events” as ATP,\(^1\)\(^-\)\(^3\)\(^-\)\(^5\)\(^-\)\(^8\) had no effect on the labeling index of BAECs. Therefore, the biochemical mechanism of the ATP mitogenic effect remains unclear. In a broader sense, our results are consistent with the emerging concept that activation of phosphoinositide turnover elicits early biochemical events generally associated with mitogenesis (Na\(^+\)-H\(^+\) antiport activation, c-fos, and c-myc expression) but is not sufficient to stimulate or maintain continuous cell proliferation.\(^20\)\(^,\)\(^21\)

The inhibition by ATP of the growth-promoting effect of bFGF might be related to the activation of protein kinase C.\(^1\) Indeed, several authors have reported that kinase C stimulation by phorbol esters inhibits the growth of endothelial cells,\(^22\)\(^-\)\(^24\) possibly as a consequence of a downregulation of bFGF receptors.\(^23\) The antagonism of bFGF by ATP in endothelial cells contrasts with a P2-receptor–mediated synergism with growth factors, such as epidermal growth factor or insulin, which has been observed in 3T3 fibroblasts and A431 keratinocytes.\(^25\) This example illustrates that the effects of ATP and adenosine on cell growth are quite variable from one cell type to the other. Both ATP and adenosine are mitogenic for thymocytes.\(^26\)\(^,\)\(^27\) On the other hand, adenosine inhibits the proliferation of hepatoma cells and fibroblasts, via an interference with pyrimidine biosynthesis.\(^28\)\(^-\)\(^30\) ATP arrests the growth of human tumor cells in vitro\(^31\) and exhibits anticancer activity in vivo.\(^32\)
The mitogenic action of adenosine on BAECs was neither mimicked by R-PIA and NECA, which are selective agonists of A₁- and A₂-receptors, respectively, nor inhibited by 8-phenyltheophylline, an antagonist of both types of receptors. Meininger and colleagues have observed a stimulatory effect of adenosine on BAEC proliferation. In their studies however, 8-phenyltheophylline was inhibitory. The reason for this discrepancy is not clear. The effect of adenosine on the labeling index of BAECs was not mimicked by DDA, a selective ligand of the inhibitory P site on adenylate cyclase; furthermore, it was reproduced by inosine and hypoxanthine, which are not active at this site. Dipyridamole could not be used to determine whether the action of adenosine is intracellular or extracellular. Indeed, it decreased the incorporation of [³H]thymidine, even in unstimulated cells (data not shown). Leitman et al have shown previously that dipyridamole decreases the proliferation rate of BAECs. Inhibition of cAMP phosphodiesterase and of nucleoside transport can both contribute to this effect. Vann and Ryan have recently reported that adenosine, as well as hypoxanthine and inosine, increases phagocytosis and decreases bactericidal killing of Staphylococcus aureus in BAECs; the pharmacological features of this action are quite similar to those characterizing the mitogenic effect that we have observed.
TABLE 2. Inhibition by Adenine Nucleotides of the Growth-Promoting Effect of Basic Fibroblast Growth Factor on Bovine Aortic Endothelial Cells

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>bFGF (10 ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cells per dish (× 10⁻⁴)</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.20±0.05</td>
<td>0.48±0.02</td>
</tr>
<tr>
<td>ATP</td>
<td>0.18±0.02</td>
<td>0.22±0.03</td>
</tr>
<tr>
<td>ATPγS</td>
<td>0.17±0.02</td>
<td>0.26±0.02</td>
</tr>
<tr>
<td>ADP</td>
<td>0.19±0.02</td>
<td>0.20±0.04</td>
</tr>
<tr>
<td>ADPβS</td>
<td>0.21±0.03</td>
<td>0.20±0.02</td>
</tr>
<tr>
<td>APPNP</td>
<td>0.18±0.02</td>
<td>0.25±0.03</td>
</tr>
<tr>
<td>APCPP</td>
<td>0.18±0.07</td>
<td>0.36±0.04</td>
</tr>
</tbody>
</table>

Values represent the cell number obtained after 3 days (mean±SD of measurements in triplicate dishes in one representative experiment out of three). bFGF, basic fibroblast growth factor; ATPγS, adenosine 5'-O-(3-thio)triphosphate; ADPβS, adenosine 5'-O-(2-thio)di phosphate; APPNP, adenosine 5'-(β,γ-imido)triphosphate; APCPP, adenosine 5'-(α,β-methylene)triphosphate. Cells were seeded at a density of 200,000 cells per dish in the presence of 2.5% fetal calf serum; after 4 hours the medium was replaced by serum-free medium. The experiment was started 24 hours later by the addition of fresh medium containing 1% fetal calf serum and the tested agents. All nucleotides were present at a 100-μM concentration. This medium, including the tested agents, was renewed every other day.

Several studies have indicated that platelets contain factors that modulate the proliferation of endothelial cells.40-46 Some of them, such as transforming growth factor-β,40 platelet factor-4,41 and thromboxane,42 are inhibitory. On the other hand, Clemenons et al44 have isolated from human serum a dialyzable factor of platelet origin, which stimulates the replication of porcine aortic endothelial cells. The recently cloned, platelet-derived endothelial cell growth factor stimulates the replication of endothelial cells in culture and exhibits angiogenic activity in vivo.45 However, other studies have shown that human umbilical vein endothelial cells and BAECs grow equally well in serum derived from plateletpoor or platelet-rich plasma.47,48 Therefore, it appears that endogenous factors, such as an autocrine stimulation by bFGF49 and true cell-contact inhibition,50 play a more significant role than platelet-derived factors in the control of endothelial cell growth. Our studies on the action of ATP, a major platelet secretory product, are in agreement with this concept, since they failed to detect a true proliferative action of ATP on cultured BAECs.

In conclusion, we have shown that ATP produces a slight but significant increase in the fraction of BAECs that initiates DNA synthesis. However, whatever the serum concentration added, ATP was unable to accelerate the proliferation of these cells and the formation of a confluent monolayer. Finally, ATP slowed down the growth of endothelial cells induced by bFGF. The physiological significance of these actions remains unclear. In any case, our results do not allow the conclusion that ATP, released from platelets aggregated on an endothelial lesion, contributes to the repair of that lesion.

Acknowledgments

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