Possible Role of Cell Surface H⁺-ATP Synthase in the Extracellular ATP Synthesis and Proliferation of Human Umbilical Vein Endothelial Cells

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Abstract
Extracellular ATP synthesis on human umbilical vein endothelial cells (HUVECs) was examined, and it was found that HUVECs possess high ATP synthesis activity on the cell surface. Extracellular ATP generation was detected within 5 s after addition of ADP and inorganic phosphate and reached a maximal level at 15 s. This type of ATP synthesis was almost completely inhibited by mitochondrial H⁺-ATP synthase inhibitors (e.g., efrapeptins, resveratrol, and piceatannol), which target the F₁ catalytic domain. Oligomycin and carbonyl cyanide m-chlorophenylhydrazone, but not potassium cyanide, also inhibited extracellular ATP synthesis on HUVECs, suggesting that cell surface ATP synthase employs the transmembrane electrochemical potential difference of protons to synthesize ATP as well as mitochondrial H⁺-ATP synthase. The F₁-targeting H⁺-ATP synthase inhibitors markedly inhibited the proliferation of HUVECs, but intracellular ATP levels in HUVECs treated with these inhibitors were only slightly affected, as shown by comparison with the control cells. Interestingly, piceatannol inhibited only partially the activation of Syk (a nonreceptor tyrosine kinase), which has been shown to play a role in a number of endothelial cell functions, including cell growth and migration. These findings suggest that H⁺-ATP synthase-like molecules on the surface of HUVECs play an important role not only in extracellular ATP synthesis but also in the proliferation of HUVECs. The present results demonstrate that the use of small molecular H⁺-ATP synthase inhibitors targeting the F₁ catalytic domain may lead to significant advances in potential antiangiogenic cancer therapies.

Introduction
Angiogenesis, the formation of new vessels from preexisting microcapillaries, is an essential step in many physiological and pathological processes, including embryonic development, wound healing, and tumor growth (1). Several recent studies have shown that both tumor growth and metastasis to distant organs are dependent on angiogenesis (1–8). Tumors often overexpress several proangiogenic molecules, including members of the fibroblast growth factor and vascular endothelial growth factor (VEGF) families (9–11); excessive angiogenesis plays a role in the pathology of cancer. Antiangiogenic therapy is therefore currently believed to be a promising approach to the development of new treatments for a variety of types of cancer. Angiostatin, a proteolytic fragment of plasminogen, has been identified and characterized as a potent inhibitor of angiogenesis (4). Angiostatin inhibits proliferation (12), blocks migration (13), and induces apoptosis (14) of endothelial cells. It has been reported that angiostatin can stop or slow cancer growth in mice apparently by preventing the formation of new blood vessels needed to nourish growing tumors (1). Although its mechanism of action remains unclear, a surprising target of angiostatin has recently been reported by Moser et al. (15) who demonstrated that angiostatin binds to the α- or β-subunits of mitochondrial H⁺-ATP synthase on the surface of human umbilical vein endothelial cells (HUVECs). Importantly, it has been suggested that the binding of angiostatin to the subunit(s) of H⁺-ATP synthase is related to angiostatin-induced down-regulation of endothelial cell proliferation and migration. However, that study did not reveal whether or not all of the components of the H⁺-ATP synthase complex were present on the endothelial cell surface, nor the small molecular H⁺-ATP synthase inhibitors targeting the F₁ catalytic domain also inhibit the HUVEC proliferation.

Recently, many polyphenolic phytochemicals such as piceatannol and resveratrol have been shown to inhibit mitochondrial H⁺-ATP synthase by targeting the F₁ catalytic domain (16, 17). Piceatannol (trans-3', 4', 3'-tetradeoxystilbene, 3-hydroxyresveratrol) and resveratrol, an analogue of piceatannol, have been shown to possess cancer chemopreventive activity (16, 18). Using a solubilized and purified F₁-ATPase preparation, piceatannol and resveratrol were shown to target the F₁ catalytic domain of H⁺-ATP synthase, although they had little effect on Na⁺,K⁺-ATPase activity in the porcine cerebral cortex. On the other hand, several H⁺-ATP synthase inhibitors have been described, including efrapeptins (F₁-targeting inhibitors) and oligomycin (a F₀-targeting...
inhibitor; 19–23). Efrapeptins are a family of closely related peptide antibiotics from the soil hyphomycete Tolypocladium (19); they are known to inhibit photophosphorylation in plants and bacteria (20, 21) and oxidative phosphorylation in mitochondria (22, 23). The binding pocket for efrapeptin was shown to be localized in the α, β, and γ subunits of the F1 catalytic domain of bovine H+-ATPase based on the structure of the F1-ATPase-efrapeptin complex determined by X-ray crystallography (24). It has been suggested that efrapeptins inhibit F1-ATPase by blocking the conversion of the non-nucleotide binding conformation of the β subunit to a nucleotide binding conformation as would be required by an enzyme mechanism involving cyclic interconversion of catalytic sites (24). Therefore, these properties of phytochemicals and efrapeptins are of particular interest for characterizing enzyme mechanism involving cyclic interconversion of catalytic sites (24). We next examined the effects on extracellular ATP synthesis of mitochondrial H+-ATP synthase inhibitors targeting the F1 catalytic domain. In this study, we used commercially available efrapeptins, which are a mixture of two related peptides. As shown in Fig. 2, efrapeptin DE (consisting of at least 50% efrapeptin D and 20% efrapeptin E as well as other forms) and efrapeptin FG (consisting of at least 60% efrapeptin F and 15% efrapeptin G as well as other forms) dose-dependently inhibited extracellular ATP synthesis by HUVECs. The IC50 value for efrapeptin DE was about 0.3 μM, but the extracellular ATP synthesis was inhibited only by about 60% even in the presence of high concentrations of efrapeptin FG. On the other hand, these efrapeptins had no significant effect on intracellular ATP synthesis by HUVECs. Similarly, piceatannol and resveratrol also dose-dependently inhibited extracellular ATP synthesis by HUVECs (Fig. 3). At 20 μM, piceatannol and resveratrol inhibited extracellular ATP synthesis by about 65% and 85%, respectively, and IC50 values for piceatannol and resveratrol were about 1.5 and 2 μM, respectively. However, these inhibitors had no significant effect on intracellular ATP synthesis by HUVECs (data not shown). Importantly, the IC50 values of these inhibitors as regards the inhibition of extracellular ATP synthesis are in good agreement with those for the inhibition of the ATPase activity of purified H+-ATP synthase (16). These results thus confirmed the presence of mitochondrial H+-ATP synthase on the extracellular surface of HUVECs.

**Inhibition of Extracellular ATP Synthesis on HUVECs by F1-Targeting H+-ATP Synthase Inhibitors**

Although F1-targeting H+-ATP synthase inhibitors almost completely inhibited extracellular ATP production by HUVECs, oligomycin, known to inhibit H+-ATP synthase by targeting the F0 subcomplex, inhibited only about 40% as shown in Fig. 4. On the other hand, m-chlorophenylhydrazone (CCCP), a mitochondrial uncoupler, almost completely inhibited extracellular ATP production by HUVECs, suggesting that cell surface ATP synthase employs the transmembrane electrochemical potential difference of protons to synthesize ATP as well as mitochondrial H+-ATP synthase. These inhibitors had no effect on intracellular ATP production. Potassium cyanide (KCN), an inhibitor of cytochrome

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**Results**

**Extracellular ATP Synthesis on HUVECs**

When the extracellular and intracellular ATP contents were determined after incubation of the HUVECs in the presence of ADP and inorganic phosphate (Pi), it was found that the extracellular ATP content had greatly increased within 5 s, reached a maximal level at 15 s, and then slightly decreased (Fig. 1). After 15 s of incubation, the extracellular ATP content increased ~14 times. However, the intracellular ATP content did not change significantly during the incubation period. Because the cells were intact during the incubation, as determined by trypan blue exclusion assay (data not shown), these results suggest that the extracellular ATP detected was not derived from intracellular pools and that ATP-synthesizing enzyme(s) exists on the surface of HUVECs.

**Effects of Oligomycin, m-Chlorophenylhydrazone, and Potassium Cyanide on Extracellular ATP Synthesis by HUVECs**

Although F1-targeting H+-ATP synthase inhibitors almost completely inhibited extracellular ATP production by HUVECs, oligomycin, known to inhibit H+-ATP synthase by targeting the F0 subcomplex, inhibited only about 40% as shown in Fig. 4. On the other hand, m-chlorophenylhydrazone (CCCP), a mitochondrial uncoupler, almost completely inhibited extracellular ATP production by HUVECs, suggesting that cell surface ATP synthase employs the transmembrane electrochemical potential difference of protons to synthesize ATP as well as mitochondrial H+-ATP synthase. These inhibitors had no effect on intracellular ATP production. Potassium cyanide (KCN), an inhibitor of cytochrome
c oxidase, had no significant effect on either extracellular or intracellular ATP production by HUVECs, suggesting that intracellular mitochondria were not involved in the extracellular ATP synthesis observed in this study. When Pi was omitted from the reaction mixture, extracellular ATP production (+ADP, +Pi) was reduced by about 28% of the control (+ADP, +Pi). Importantly, the extracellular ATP synthesis determined in the absence of Pi was not inhibited significantly by oligomycin, CCCP, piceatannol, or efrapeptin DE (Fig. 4B), suggesting that this activity was mediated by another ectonucleotide kinase such as adenylate kinase or nucleoside diphosphate (NDP) kinase (described below) but not by H+-ATP synthase. Taken together, these results confirm that extracellular ATP synthesis determined in the presence of ADP and Pi was mediated mostly by H+-ATP synthase, but it is not clear whether H+-ATP synthase accounts for the bulk of the extracellular ATP synthesis because the medium is not truly phosphate free.

**Effects of Piceatannol on the Activities of Adenylate Kinase and NDP Kinase**

Recently, two different types of ectonucleotide kinases, adenylate kinase and NDP kinase, were identified on the surface of HUVECs (26). Thus, it is also possible that the extracellular ATP synthesis by HUVECs observed in this study was mediated by these two enzymes via phosphotransfer reactions, as described by Yegutkin et al. (26). However, NDP kinase activity was very low under our assay conditions, as shown in Table 1. This result was likely due to the absence of γ-terminal phosphate donors such as GTP, ITP, UTP, and ATP, which are required for the interconversion of nucleoside triphosphate and NDP through NDP kinase under the present assay conditions. Next, we examined whether adenylate kinase was involved in the type of extracellular ATP synthesis detected in the present study; to this end, we determined the effect of piceatannol on the activity of purified adenylate kinase. As

**FIGURE 2.** Effects of efrapeptins DE and FG on ATP synthesis by HUVECs. Confluent HUVECs were preincubated for 1 min with or without the indicated concentrations of efrapeptins, and the ADP solution was added to the culture. After 15 s, the ATP content in the extracellular medium and in the intracellular pools was determined as described in "Materials and Methods." Points, relative values derived from three independent experiments performed with triplicate samples; bars, SD. *, P < 0.05 and **, P < 0.01 versus without inhibitors.

**FIGURE 3.** Effects of piceatannol and resveratrol on extracellular ATP synthesis by HUVECs. Extracellular ATP production was determined as described in Fig. 2. Points, mean of three independent experiments performed with triplicate samples; bars, SD. *, P < 0.05 versus without inhibitors. Ethanol (0.1%) was used as a solvent vehicle for the results of resveratrol.
shown in Table 1, piceatannol had little effect on the activity of adenylate kinase, indicating again that the extracellular ATP synthesis observed in this study was mediated primarily by H⁺-ATP synthase on the cell surface.

**Inhibition of Proliferation of HUVECs by F₁-Targeting H⁺-ATP Synthase Inhibitors**

We next performed a cell proliferation assay in the presence of F₁-targeting H⁺-ATP synthase inhibitors. As shown in Fig. 5, all of the inhibitors potently inhibited the growth of HUVECs in a dose-dependent manner. Efrapeptin DE was the most potent of the inhibitors, and its IC$_{50}$ value was 0.5 µM. Resveratrol and piceatannol were slightly less active than efrapeptin, and the IC$_{50}$ values of resveratrol and piceatannol were 2–5 and <10 µM, respectively.

**Effect of Piceatannol on the Activation and Expression of Syk on HUVECs**

Piceatannol has been used as a specific inhibitor of Syk (a nonreceptor tyrosine kinase; 27–30), which is expressed in a

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**Table 1. Effect of Piceatannol on the Activity of Adenylate Kinase and NDP Kinase**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Enzyme Activity (nmol ATP generated/min)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Adenylate Kinase</td>
</tr>
<tr>
<td>None</td>
<td>9.63 ± 2.63</td>
</tr>
<tr>
<td>Piceatannol (4 µM)</td>
<td>7.94 ± 1.17</td>
</tr>
</tbody>
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Note: Purified adenylate kinase (5 units) and NDP kinase (5 units) were preincubated with 4-µM piceatannol, and ATP synthesis was initiated by the addition of 200-µM ADP. After 15 s, ATP generated in the reaction mixture was determined as described in "Materials and Methods." Data are means ± SD of two independent experiments performed in triplicate.
wide variety of cells and recently has been shown to play a critical role in a number of endothelial cell functions, including morphogenesis, cell growth, and migration (31). Phosphorylation of Tyr525/526 in the activation loop of human Syk has been demonstrated to be essential for Syk function (32). To clarify whether inhibition of Syk is implicated in piceatannol-induced growth inhibition of HUVECs, we next examined the activation state of Syk in HUVECs exposed to piceatannol. HUVECs were growth factor starved for 24 h in endothelial cell basal medium-2 (EBM-2) containing 2% FCS and were stimulated by changing the medium to EBM-2 (+) containing 2% FCS and growth factors with or without piceatannol. The total cell lysates were then subjected to immunoblotting analysis using anti-phospho-Syk (p-Syk) antibody, which recognizes phosphorylated Tyr525/526 of Syk. As shown in Fig. 6B, proliferative stimulation by addition of growth factor-supplemented EBM-2 increased the level of p-Syk in HUVECs after 2 h of incubation. Activation of Syk was observed even after 24 h of incubation. However, piceatannol at 20 μM had little effect on the activation of Syk during the incubation period in which piceatannol, at the same concentration, inhibited the growth of HUVECs by about 80% (Fig. 5). Piceatannol had no effect on the total Syk protein level.

**Discussion**

Moser et al. (25) detected cell surface-associated ATP synthesis on HUVECs and demonstrated that extracellular

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**FIGURE 5.** Effects of efrapeptin DE, resveratrol, and piceatannol on the proliferation of HUVECs. HUVECs (5 × 10⁴ cells/ml) were cultured in the presence or absence of efrapeptin DE (A), resveratrol (B), and piceatannol (C) for the indicated times. A. None (○), 0.5 μM (●), 3 μM (□), and 15 μM (▲). B. Ethanol (0.1%) solvent vehicle (○), 2 μM (●), 5 μM (□), and 20 μM (▲). C. None (○), 4 μM (●), 10 μM (□), and 20 μM (▲). Cell numbers were determined by MTT assay as described in “Materials and Methods.” Points, mean of three independent experiments performed in duplicate; bars, SD. *, P < 0.05; **, P < 0.01, and ***, P < 0.001 versus without inhibitors.

**FIGURE 6.** Effects of H+-ATP synthase inhibitors on intracellular ATP levels in HUVECs (A) and the effect of piceatannol on the activation and expression of Syk on HUVECs (B). A. HUVECs were cultured in EBM-2 (+) for 24 h in the presence or absence of the indicated concentrations of H+-ATP synthase inhibitors. After incubation, the intracellular ATP content was determined as described in “Materials and Methods.” Points, mean of three independent experiments performed with triplicate samples; bars, SD. Ethanol (0.1%) was used as a solvent vehicle for the results of resveratrol. B. HUVECs were cultured in EBM-2 without growth factors for 24 h. After incubation, cells were stimulated by replacing the medium with EBM-2 (+) for the indicated times in the presence or absence of the indicated concentrations of piceatannol for 2 or 24 h. Cell lysates were prepared, resolved on SDS-PAGE, and subjected to Western blotting as described in “Materials and Methods.” Membranes were incubated with anti-Syk, anti-p-Syk, and anti-β-actin polyclonal antibodies. Protein bands were visualized by enhanced chemiluminescence according to the manufacturer’s instructions. The expression of β-actin was used as a loading control. The relative values of p-Syk and Syk (in arbitrary units), normalized for differences in loading, are indicated under each lane. Data are representative of three independent experiments with similar results.
ATP synthesis was inhibited by angiostatin as well as by
antibodies directed against the α and β subunits of
mitochondrial H+-ATP synthase. Their study also showed that
angiostatin binds to purified bovine F₁,F₂ATP synthase and
inhibits the ATP hydrolytic activity of purified F₁,F₂ATP
synthase. Based on these results, it was suggested that H+-
ATP synthase is the primary target for angiostatin. Moreover,
they hypothesized that the antiangiogenic activity of
angiostatin was mediated through the inhibition of the
enzymatic activity of cell surface H+-ATP synthase. However,
the activity of extracellular ATP synthesis by HUVECs was
extremely low (about 40 pmol ATP produced/min/10⁶ cells) in
contrast with our present results; in fact, the activity
demonstrated in their study was the lowest among the
ectonucleotide kinases thus far reported as being active on
HUVECs (26), as described below. In addition, the inhibitory
effect of angiostatin on cell surface-associated ATP synthesis
was not comparable with its inhibitory effect on cell
proliferation (i.e., angiostatin more effectively inhibited ATP
synthesis than it inhibited cell proliferation; 15, 25). In
contrast, we showed in the present study that HUVECs possess high ATP synthesis activity on the cell surface (about
50 nmol ATP produced/min/10⁶ cells, as described below);
this observed activity was the highest among the ectonucleo-
tide kinases reported to date and was strongly inhibited by H+-
ATP synthase inhibitors targeting the F₁ catalytic domain (i.e.,
the efrapeptins, resveratrol, and piceatannol). We also showed
that these inhibitors potently inhibited the proliferation of
HUVECs. It is of note that the IC₅₀ values of these inhibitors
(with the exception of that of piceatannol) showing the
inhibition of cell surface-associated ATP synthesis were in
good agreement with those showing the inhibition of HUVEC
proliferation (this study) and ATPase activity of purified
mitochondrial H+-ATP synthase (16). At present, the reason
for the large differences between Moser et al.’s results and our
studies as regards extracellular ATP synthesis activity remains
unclear, but differences in culture conditions between Moser et
al.’s and our assay methods are possible. In their study,
HUVECs were incubated overnight without FCS to allow the
cells to become quiescent, and confluent HUVECs were
equilibrated into DMEM/Ham’s F-12 (1:1) medium containing
10-mM potassium phosphate (cell surface ATP assay). On the
other hand, in our experiments, HUVECs were incubated in
EBM-2 supplemented with 2% FCS and growth factors
including VEGF and basic fibroblast growth factor (bFGF),
and confluent HUVECs were used. Thus, the activity or
expression level of H+-ATP synthase on the cell surface may
be influenced by incubating the cells without FCS or growth
factors. The activity of the enzyme also may be influenced by
the medium used. Interestingly, Wahl et al. recently reported that extracellular pH is critical parameter in the evaluation of angiostatin’s activity (33, 34). They showed that the antiangiogenic activity of angiostatin on endothelial cells is enhanced in culture when the microenvironmental extracellular pH is reduced to levels similar to that of many tumors (pH 6.7). In the present study, pH of media was 7.3–7.4 (cell surface ATP assay) and 7.2–7.5 (cell proliferation) during the incubation. Whether pH change in the media is a critical parameter in the activities of H+-ATP synthase inhibitors targeting the F₁ catalytic domain is not known, but further studies should be directed to understand the regulatory
mechanism of the expression and activity of H+-ATP synthase on the cell surface.

Recent studies have demonstrated that H+-ATP synthase is a
common target site for aglycone isoflavones (i.e., genistein,
biochanin A, and daidzein) from soybeans, gallate esters of
catechins from many sources, and several other polyphenolic
compounds at low micromolar concentrations similar to those
used in cancer chemopreventive studies as well as resveratrol
and piceatannol; however, genistein most likely targets F₀
(16, 17, 35–37). Piceatannol has been reported to inhibit tumor
cell growth by the inhibition of protein tyrosine kinases, Syk in
particular, which is thought to be a potential target for
angiogenesis inhibition of tumor growth when it is present in
concentrations of 10–200 µM (38, 39). Resveratrol has also
been shown to inhibit the activity of cyclooxygenase-1, which
catalyzes the conversion of arachidonic acid to proinflamma-
tory substances such as prostaglandins; accordingly, resveratrol
has already been shown to be involved in tumor cell growth and
immunosuppression (40, 41). Thus, it is possible that
piceatannol or resveratrol crosses the lipid bilayer
membrane and inhibits the activation of Syk or cyclooxygenase-
ase-1 activity, thereby resulting in the inhibition of cell
growth. However, piceatannol was shown to have little effect
on the activation and expression of Syk in HUVECs (Fig. 6B).
Furthermore, piceatannol, resveratrol, and efrapeptins had little
effect on intracellular ATP levels in HUVECs (Fig. 6A). Although the effect of resveratrol on the activity of cyclo-
oxynase-1 in HUVECs remains to be examined, these results
suggest that these H+-ATP synthase inhibitors cannot readily
cross cell membranes and that the target molecule(s) for these
inhibitors, including H+-ATP synthase, also exists on the cell
membrane. These findings are of particular importance from the
point of view of demonstrating the antitumor effects of stilbene
phytochemicals such as piceatannol and resveratrol as well as
those of other polyphenolic compounds.

The molecular mechanism that demonstrates how picea-
tannol and resveratrol act to inhibit HUVEC proliferation is
not known. As shown in this study, however, cell surface
H+-ATP synthase on HUVECs is active in ATP synthesis.
This result shows that this enzyme participates in the
regulation of extracellular nucleotide concentrations. Thus,
it might be possible that H+-ATP synthase inhibitors inhibit
the development of new blood vessels (angiogenesis) by
regulating extracellular nucleotide concentrations because
purines or purine nucleotides have been shown to be
significant mitogenic, chemotactic, and angiogenic factors
(42–45). The role of protein kinases in angiogenesis is also
well established, but Syk seems not to be a potential target
for angiogenesis. Recently, we found that extracellular
signal-regulated kinase (ERK) was markedly activated when
HUVECs were stimulated by growth factors including bFGF
and VEGF and that flavone, which is shown to inhibit
HUVEC proliferation and tube formation (Arakaki et al.,
unpublished observations), almost completely inhibit the
activation of ERK. We are now investigating the effect of
H+-ATP synthase inhibitors on various signaling pathways
including ERK cascade.
Recently, Yegutkin et al. (26) demonstrated that HUVECs can synthesize ATP on the cell surface by two different ectonucleotide kinases, NDP kinase and adenylyl kinase, via phosphotransfer reactions. However, it is most likely that the extracellular ATP synthesis observed in the present study was mediated primarily by H+-ATP synthase because the NDP kinase activity was observed as being very low under our assay conditions and also because the activity of the purified adenylyl kinase was not significantly inhibited by piceatannol (Table 1). Furthermore, extracellular ATP production was greatly reduced when the rate of ATP synthesis was determined in the absence of P1 (Fig. 4). In addition, it was found that extracellular ATP synthesis determined in the absence of P1 was not significantly inhibited by oligomycin, CCCP, piceatannol, or efrapeptin DE (Fig. 4B). These results also show that extracellular ATP synthesis determined in the presence of both ADP and P1 was mediated primarily by H+-ATP synthase. Yegutkin et al. also indicated that the Vmax rates (nmol ATP produced/min/106 cells) for NDP kinase and adenylyl kinase were 26.6 ± 1.9 and 2.4 ± 0.2, respectively. Although we did not undertake a kinetic analysis of cell surface-associated H+-ATP synthase on HUVECs under the present conditions, the Vmax for this enzyme, as roughly calculated from Figs. 1 and 4, was about 68 nmol/min/106 cells when the protein concentration was assumed to be 300 µg/106 cells. Recently, we found that calf pulmonary arterial endothelial cells possess high ATP synthesis activity on the cell surface (about 50 nmol ATP produced/min/106 cells) and that H+-ATP synthase inhibitors targeting the F1 catalytic domain almost completely inhibited this ATP synthesis activity of calf pulmonary arterial endothelial cells. Thus, the ATP synthesis activity of cell surface H+-ATP synthase was the highest among the ectonucleotide kinases thus far reported, suggesting again that H+-ATP synthase on the surface of HUVECs plays an important role in the regulation of extracellular nucleotide concentrations.

H+-ATP synthase is a supramolecule composed of two structurally and functionally distinct sectors referred to as F1 and F0 (46–49). The catalytic sector, F1, is extramembranous and composed of six subunits (α, β, γ, δ, ε, and ɛ), and the loosely attached ATPase inhibitor protein (with constant stoichiometry; the energy transduction sector, F0, is largely membrane embedded and is composed of 10 subunits with constant stoichiometry (50–55). At present, it remains unknown whether all of the components of the H+-ATP synthase complex are present on the endothelial cell surface. Nonetheless, the present results strongly suggest the presence of at least a minimal number of components required for ATP synthesis on the cell surface. However, it is possible that the subunit composition of H+-ATP synthase on the surface of HUVECs at least in part differs from that of H+-ATP synthase on the mitochondrial inner membrane because extracellular ATP synthesis was not entirely inhibited by oligomycin in this study (Fig. 4). Importantly, Fig. 4 suggests that cell surface H+-ATP synthase employs the transmembrane electrochemical potential difference of protons to synthesize ATP as well as mitochondrial H+-ATP synthase because extracellular ATP synthesis was shown to be inhibited almost completely by CCCP (a mitochondrial uncoupler); this result suggests the presence of the transmembrane electrochemical potential difference of proton-producing apparatus on the cell surface. Determination of the subunit composition of the cell surface form of the H+-ATP synthase complex, as well as elucidation of the mechanism of H+-ATP synthase expression on the cell surface, will provide keys to understanding the physiological significance of cell surface H+-ATP synthase on HUVECs.

Materials and Methods

Materials

The sources of the materials used in this study are given below. Piceatannol, resveratrol, efrapeptins, adenylyl kinase purified from rabbit muscle, NDP kinase purified from bovine liver, oligomycin, carbonyl cyanide CCCP, and KCN were purchased from Sigma Chemical Co. (St. Louis, MO), and the ATP bioluminescence assay kit was obtained from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan). 3-(4,5-Dimethylimidazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Research Organics, Inc. (Cleveland, OH) and anti-β-actin monoclonal antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Resveratrol, oligomycin, and CCCP were dissolved in ethanol, and ethanol was used as a solvent vehicle. Other materials used in this study have been described previously (56, 57).

Cell Culture

Primary HUVECs obtained from Clonetics (San Diego, CA) were seeded on tissue culture dishes and cultured in EBM-2 supplemented with 2% FCS and growth factors including 10-ng/ml bFGF and 3-ng/ml VEGF [EBM-2 (+)]. Cells were purchased from Clonetics (San Diego, CA) in 0.4 ml of EBM-2 (+) and confluent HUVECs were used for cell surface ATP synthesis. Cells were rinsed once with HEPES buffer (10-mM HEPES [pH 7.4], 150-mM NaCl), and 0.2 ml of HEPES buffer containing 2-mM MgCl2 with or without H+-ATP synthase inhibitors were added into the wells. After 1 or 5 min(s), 0.2 ml of the same buffer containing 200-µM ADP, 20-mM potassium phosphate (P), and 2-mM MgCl2 were added. The cells were then incubated for 5–30 s at room temperature, and the extracellular media were removed and centrifuged; the resulting supernatants (50 µl) were used for the determination of extracellular ATP content. ATP was determined by using the ATP bioluminescence assay kit (Dainippon Pharmaceutical) according to the manufacturer’s instructions. To exclude the possibility of ATP release from intracellular pools, we also determined the intracellular ATP content, as described essentially by Yegutkin et al. (26). After removal of the medium, the residual HUVECs were lysed in 0.2 ml of 0.1-N NaOH for 10 min at room temperature, and the lysates were neutralized by the addition of the same volume of 0.1-N HCl. Aliquots (50 µl) of the cell lysates were used for the determination of intracellular ATP content, as described above.
Assay of Adenylate Kinase and NDP Kinase Activities

Purified adenylate kinase (5 units) and NDP kinase (5 units) were preincubated in 0.2 ml of reaction mixture containing 10 mM HEPES (pH 7.4), 150 mM NaCl, 2 mM MgCl₂, and 10 mM Pᵢ with or without 4 µM piceatannol for 1 min at room temperature. The reaction was started by the addition of 0.2 ml of the same buffer containing 200 µM ADP, and the reaction was terminated after 15 s by the addition of 0.1 ml of 20% trichloroacetic acid. Adenylate kinase and NDP kinase activities were determined by measuring the amount of ATP generated in the reaction mixture, as described above.

Cell Proliferation Assay

Cell proliferation was measured essentially, as described previously (56, 57). Briefly, HUVECs were plated into 24-well culture plates in 0.4 ml of EB-2 (+) at a density of 5 × 10⁴ cells/ml and were cultured at 37°C in a humidified atmosphere of 5% CO₂ in air. After 24 h, the medium was removed and the cells were cultured in 0.4 ml of EB-2 (+) with or without H⁺-ATP synthase inhibitors for 2 days. At the end of the incubation period, the medium was removed and 0.4 ml of EB-2 (+) containing MTT (0.5 mg/ml) were added. After 60 min, the medium was removed and 0.4 ml of acidified isopropanol (0.04-N HCl in isopropanol) were added to the wells to solubilize the formazan crystals. An aliquot (0.2 ml) of each sample was transferred into a 96-well plate. The absorbance of the MTT formazan products was measured at a wavelength of 570 nm, and the reference was measured at a wavelength of 630 nm.

Western Blot Analysis

Subconfluent HUVECs growing on 100-mm tissue culture dishes (Asahi Technoglass) were cultured in EB-2 without growth factors for 24 h. After incubation, cells were stimulated by replacing the medium with EB-2 (+) for the indicated times in the presence or absence of piceatannol at the indicated concentrations. Cells were washed with PBS, collected, and lysed in radioimmunoprecipitation assay buffer (10 mM Tris-HCl [pH 7.4], 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 10 µg/ml leupeptin, 0.1 mM trypsin inhibitor unit/ml aprotinin, 1 mM vanadate, and 100 mM NaF). Cell lysates were resolved by SDS-PAGE and subjected to Western blotting, as described previously. Protein bands were detected using horseradish peroxidase-conjugated anti-rabbit IgG antibody.

Statistical Analysis

Experimental and control samples used in the functional assays were compared for statistical significance by using Student’s t test. P < 0.05 was considered as significant.

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Molecular Cancer Research

Possible Role of Cell Surface H⁺-ATP Synthase in the Extracellular ATP Synthesis and Proliferation of Human Umbilical Vein Endothelial Cells

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