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Piceatannol, a Stilbene Phytochemical, Inhibits Mitochondrial F0F1-ATPase Activity by Targeting the F1 Complex

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Piceatannol is a stilbene phytochemical from the seeds of *Euphorbia lagascae,* previously identified as an antileukemic principle. Piceatannol is considered an inhibitor of several tyrosine kinases. We recently reported that resveratrol, another stilbene phytoalexin from grape seeds, was an inhibitor of ATP synthase. Here, we demonstrated that piceatannol potently inhibited the rat brain mitochondrial F0F1-ATPase activity in both solubilized and submitochondrial preparations (IC50 of 8–9 μM), while having relatively small effect on the Na^+, K^+-ATPase activity of porcine cerebral cortex (no effect up to 7 μM). Piceatannol inhibited the ATPase activity of the purified rat liver F1 with IC50 of about 4 μM, while resveratrol was slightly less active (IC50 of about 14 μM). Our results indicate that piceatannol and resveratrol inhibit the F-type ATPase by targeting the F1 sector, which is located to the inner membrane of mitochondria and plasma membrane of normal endothelial cells and several cancer cell lines. This mechanism could potentially contribute to the multiple effects of these chemopreventive phytochemicals.

Piceatannol (*trans*-3,4,3′,5′-tetrahydroxystilbene, 3-hydroxyresveratrol), a natural product and an analogue of resveratrol, was initially identified from the heartwood of *Vouacapoua* species (1). Later, it was identified as an antileukemic agent from the seeds of *Euphorbia lagascae* (2), which have been used widely in folk medicine for treating tumors, cancers, and warts (3). Screening of natural products which had previously been demonstrated to have antitumor activity identified piceatannol as an inhibitor of protein-tyrosine kinases (PTK), p40 (a fragment of $p72^{Syk}$, a non-receptor tyrosine kinase) and $p56^{lck}$ (4). On the other hand, piceatannol did not affect the activity of the catalytic subunit of bovine heart cAMP-dependent protein kinase (a protein serine kinase) (4). Piceatannol is a competitive inhibitor of p40 with respect to angiotensin I, the substrate (Ki = 15 μM), and it is noncompetitive with respect to ATP. It is also a weaker inhibitor of another PTK, Lyn (IC50 of 100 μM) (5). Several analogues of piceatannol have been synthesized with different bioactivities and tyrosine kinase inhibitory activities (5, 6).

Since the initial discovery of the inhibitory effect, piceatannol has been used as a specific inhibitor to reveal the involvement of PTK, especially $p72^{Syk}$ (Syk), in concentrations of 10–200 μM (e.g., 7, 8). However, others have found that piceatannol is also an inhibitor (IC50 of 3–19 μM) for several serine/threonine-specific protein kinases including rat brain Ca^{2+}- and phospholipid-dependent protein kinase (PKC) (e.g., 9).

Piceatannol belongs to a group of phytoalexins with stilbene structures. Recently, we have shown that, resveratrol, a stilbene phytoalexin that is enriched in red wines, inhibited the activity of mitochondrial ATPase/ATP synthase (F0F1-ATPase or F-type ATPase) from 0.7–70 μM with IC50 of 12–21 μM (10). The F-type ATPase is ubiquitously localized in the inner membrane of the mitochondria and is responsible for the synthesis of ATP from ADP in the oxidative phosphorylation pathway (11). The enzyme consists of a large multisubunit enzyme (about 500 kDa) made up of two major complexes, F0 and F1 (11). F1 is a water-soluble catalytic complex made up of five subunits ($\alpha_3\beta_3\gamma\delta\epsilon$), with the catalytic site located on the β-subunit. F0 is made up of several integral membrane proteins that are part of proton channels (a, b, c, d) as well as F6 and oligomycin sensitivity-conferring protein (OSCP) that contribute to the stalk region between F0 and F1. Fur-

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Abbreviations used: IC50, concentration of half-maximal inhibition; Ki, inhibition constant; Km, Michaelis constant; PTK, protein-tyrosine kinase; OSCP, oligomycin sensitivity-conferring protein; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SD, standard deviation.

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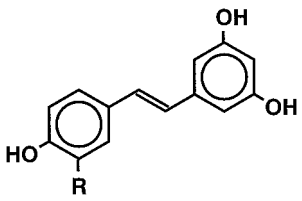

FIG. 1. The chemical structures of piceatannol (R = OH, a plant stilbene from the seeds of *Euphorbia lagascae*) and resveratrol (R = H, a plant stilbene from grapes).

thermore, a native peptide is also bound to the F1 under the deenergized condition and inhibits the ATPase activity but does not affect ATP synthase activity of the enzyme (called F1 inhibitor protein, IF1). Recent studies indicate that the β-subunit of the enzyme is also localized on the plasma membrane of three human tumor cell lines (12). Moreover, both α and β subunits of this enzyme are also localized on the plasma membrane of normal endothelial cells and are target sites for angiostatin, a proteolytic fragment of plasminogen and a potent inhibitor of angiogenesis and endothelial cell migration and proliferation (13). In this report, we have identified piceatannol as a potent inhibitor of the F0F1-ATPase. Using a purified F1-ATPase preparation, the effect of piceatannol and resveratrol was shown to target the F1 part of the F0F1-ATPase. This property is especially interesting in view of the antitumor effect of these compounds (Fig. 1).

MATERIALS AND METHODS

Materials. PD-10 columns (Sephadex G-25M) were from Pharmacia Biotech Inc. (Piscataway, NJ). Precasted 4–20% gradient gel (1 mm thick) and the protein standard markers were obtained from Bio-Rad (Hercules, CA). Fast stain was from Zoion Research, Inc. (Allston, MA). Piceatannol was from CalBiochem, Inc. 4-(2-Aminoethyl)-benzenesulfonyl fluoride (AEBSF) was from Boehringer Mannheim (Indianapolis, IN). Oligomycin (mixture of A, B, C) was purchased from Aldrich (Milwaukee, WI), and prepared in methanol as a stock solution (1 mg/ml). Efrapeptin was kindly provided by Dr. J. Clemens (Eli Lilly, Indianapolis, IN) and dissolved in sterile distilled water with a concentration of 0.1 mM. All other chemicals and reagents were from Sigma Chemical Co. (St. Louis, MO). Phytochemicals and steroids tested were dissolved in 100% ethanol at stock concentration of 10 mM.

Animals. Adult Sprague-Dawley rats were maintained on a 14:10 hr light/dark cycle (lights on at 0700) with food and water available *ad lib.* Animals were taken care of in accordance with federal and institutional guidelines.

Preparation of submitochondrial particles and solubilized mitochondrial fraction. Adult female rats were sacrificed by rapid decapitation. Rat brains were removed quickly and homogenized in ice-cold Tris buffer (50 mM Tris·HCl/120 mM NaCl/5 mM KCl/1 mM $MgSO_4$/1 mM $CaCl_2$/10% glycerol/0.5 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF)/0.1 mM bacitracin, pH 7.4 at 4°C). Mitochondrial fraction, 1% digitonin-solubilized brain mitochondrial fractions that possessed high F0F1-ATPase activity, and submitochondrial particles were obtained as described earlier (14). Proteins were assayed by the method of Bradford (15) with bovine serum albumin as standard and either kept at 4°C for use in the same day or stored at −80°C for later use.

Purification of liver F1-ATPase. Rat liver mitochondria were isolated from overnight-fasted adult male rats by differential centrifugation in an isolation medium containing 250 mM sucrose, 20 mM Tris, pH 7.4, and 0.1% bovine serum albumin (16). Freshly isolated mitochondria (100 mg/ml) were used to obtain the inverted inner membranes and stored frozen at −80°C for later use (17). The liver F1-ATPase was then isolated from the inverted inner membranes according to Williams *et al.* (17) with slight modification. Briefly, the inverted inner membrane vesicles were thawed and sedimented at 125,000 × g for 30 min at 4°C. The pellet was washed by resuspension in sucrose/Tris buffer plus 5 mM EDTA to 20 mg/ml and centrifuged as above. The inverted inner membranes were resuspended in sucrose/Tris/EDTA buffer to 10 mg/ml at room temperature and ATP was added to final concentration of 100 μM. Five min later, chloroform equaled to half the volume of the inner membrane was added. The mixture was shaken for 20 sec at room temperature and immediately centrifuged for 10 min in a laptop centrifuge at room temperature to separate the aqueous and organic phases. The upper aqueous phase was carefully removed and centrifuged at 125,000 × g for 40 min at room temperature. The clear supernatant (F1) was then removed and exchanged at room temperature with stabilizing buffer (250 mM potassium phosphate, 5 mM EDTA, pH 7.5) by a PD-10 column and collected in 1 ml fraction. The protein-containing fractions were assayed for the ATPase activity and the purity of F1 was checked by SDS-PAGE. Aliquots of 100 μl of the most active fraction were frozen on dry ice, lyophilized, and stored desiccate at −20°C. The lyophilized F1 was later reconstituted in deionized water at room temperature and used for ATPase activity assay (kept at room temperature).

Assay for ATPase activity. The ATPase activity of solubilized mitochondrial fraction, submitochondrial fraction, and purified F1 preparation was measured spectrophotometrically at 340 nm by coupling the production of ADP to the oxidation of NADH via the pyruvate kinase and lactate dehydrogenase reaction as described previously (14). The reaction mixture contained in a final volume of 0.7 ml at 30°C included the following: 100 mM Tris (pH 8.0), 4.0 mM MgATP, 2 mM $MgCl_2$, 50 mM KCl, 0.2 mM EDTA, 0.23 mM NADH, 1 mM phosphoenol pyruvate, 1.4 unit of pyruvate kinase, 1.4 unit of lactate dehydrogenase and about 50–135 μg proteins for unpurified preparations or 0.8–1.2 μg for purified preparation. For submitochondrial preparations, antimycin A (2 μg/ml) was also included to inhibit the NADH oxidase activity. The F-type ATPase activity was determined in the presence of oligomycin, a F0-targeting inhibitor, or efrapeptin, a specific and potent F1-targeting inhibitor (18).

The activity of the Na^+, K^+-ATPase from porcine cerebral cortex was measured similarly by the coupled assay as described above except 100 mM NaCl was included.

Data are expressed as means ± standard deviation (SD) when 3 or more trials were performed.

SDS-PAGE. The samples were treated at 95°C for 3 min in the presence of 1 × reducing sample buffer. The samples were cooled and then applied to a 4–20% precast polyacrylamide gradient minigel, and SDS-PAGE was performed at 200 V for 35 min according to Laemmli (19). The gel was stained with Fast stain.

RESULTS AND DISCUSSIONS

Inhibition of F0F1-ATPase by Piceatannol

Initial studies were carried out to examine the effect of piceatannol on the F0F1-ATPase activity using both a solublized mitochondrial preparation and a submito-

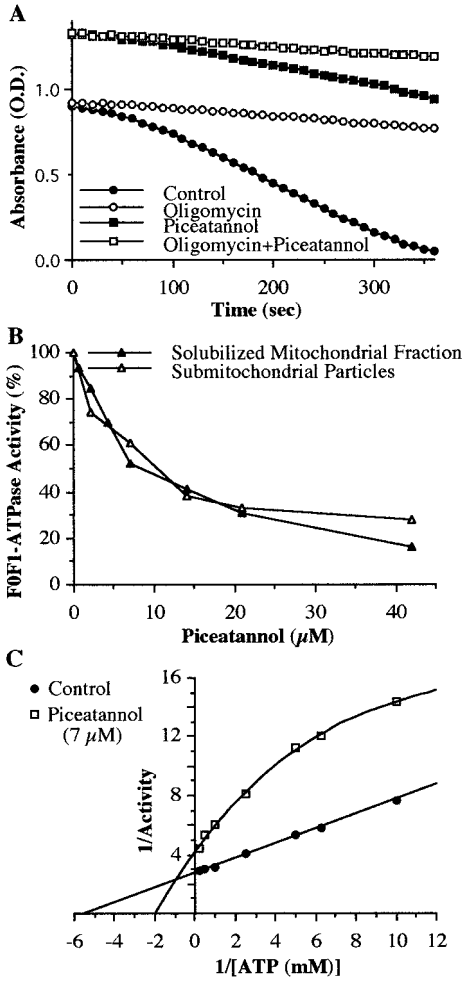

FIG. 2. Effect of piceatannol on the F0F1-ATPase activity of rat brain mitochondrial preparations. (A) Typical examples of spectrophotometric read-out showing the effect of piceatannol (21 μM) on the F0F1-ATPase activity of digitonin-solubilized mitochondrial preparation (50 μg/0.7 ml). The effect of piceatannol was abolished in the presence of oligomycin (7 μg/ml) and the oligomycin-insensitive part of the total ATPase activity was not affected. Piceatannol had significant absorbance at 340 nm, at which the ATPase activity was measured, but it did not interfere with the ATPase assay. (B) Effect of piceatannol (0.7–42 μM) on the F0F1-ATPase activity of both digitonin-solubilized fraction and submitochondrial particles. The data are expressed as percentage inhibition. The control F0F1-ATPase activities for both preparations were 0.329 $\pm$ 0.004 and 0.088 $\pm$ 0.002 μmol ATP hydrolyzed/min/mg protein (n = 3 for both), respectively, and are considered as 100%. (C) Lineweaver-Burk plots of the enzyme activity of digitonin-solubilized mitochondrial preparation at different concentrations of ATP (0.1–4 mM) in the absence and presence of piceatannol (7 μM). The control is a linear plot giving us an apparent Km of about 0.18 mM and maximal enzyme specific activity of about 0.366 μmol/min/mg protein.

chondrial particle preparation of rat brain (Fig. 2). Earlier studies have shown that more than 80% of total ATPase activity in these preparations were sensitive to efrapeptin and oligomycin, specific inhibitors of mitochondrial F0F1-ATPase (10, 14 and Fig. 2A). Piceatannol at 21 μM inhibited the solubilized mitochondrial ATPase activity and its effect was abolished in the presence of oligomycin at 7 μg/ml (Fig. 2A). The oligomycin-insensitive part (15.0%) of the total activity was not significantly affected, since in the presence of both oligomycin and piceatannol the activity remained was 14.2% (Fig. 2A). Dose dependent studies indicated that piceatannol inhibited the F0F1-ATPase activity in both preparations with IC50 of about 8–9 μM (Fig. 2A, 2B), indicating that piceatannol is a more potent inhibitor than resveratrol (IC50 of 12–21 μM) as reported before (10). The coupled enzymes used in the assay (pyruvate kinase and lactate dehydrogenase) were not affected, since piceatannol did not change the responses induced by ADP (0.2 mM) (100.0 $\pm$ 1.3% for control and 100.2 $\pm$ 0.8% for 5 μM piceatannol, each with 3 trials).

The inhibition of F0F1-ATPase activity of solubilized preparation by piceatannol (7 μM) was further examined at different concentrations of ATP (0.1–4 mM) (Fig. 2C). When the data are expressed in double reciprocal plots, the control without piceatannol shows a linear curve with apparent Km for ATP of 0.18 mM and maximal enzyme specific activity of about 0.366 μmol ATP hydrolyzed/min/mg protein. This Km is very similar to the values determined in purified and membrane-bound porcine heart F1 (0.17–0.28 mM) (20). However, in the presence of piceatannol (7 μM), a curved plot resulted. Therefore, it seems that piceatannol behaves like a mixed-type inhibitor.

Effect of Piceatannol and Resveratrol on the F1-ATPase Activity

To investigate if piceatannol inhibited the F0F1-ATPase activity by targeting the F1 or F0 part of the enzyme, a purified rat liver F1 preparation was used in this study (Fig. 3). When F1 was exchanged to the stabilizing solution by a PD-10 column, the proteins appeared in fractions 3 and 4 (Fig. 3A). The total proteins obtained in this experiment were 214 μg from about 4 mg of the inverted inner membrane. The total recovery of proteins was thus about 5%. Analysis of the purified proteins by 4–20% gradient SDS-PAGE indicates that this F1 preparation was more than 90% pure with mainly α, β, γ, δ subunits (the presence of ϵ subunit is uncertain) (Fig. 3B). The F0 subunits and OSCP were apparently absent. Several additional bands appeared at 41, 26, and 14 kDa, but do not seem to interfere with the specificity. The ATPase assay indicated that the purified F1 was highly active with specific ATPase activity of about 20 μmol ATP hydrolyzed/min/mg protein when initially isolated, and it was sensitive to efrapetin (about 97% inhibition), but not oligomycin, a F0-targeting inhibitor (Fig. 3C and Table 1). Piceatannol dose-dependently (0.5 to 50 μM) inhibited the ATPase activity of the purified F1 with IC50 of 4 μM (Fig. 3D). We then tested resveratrol and

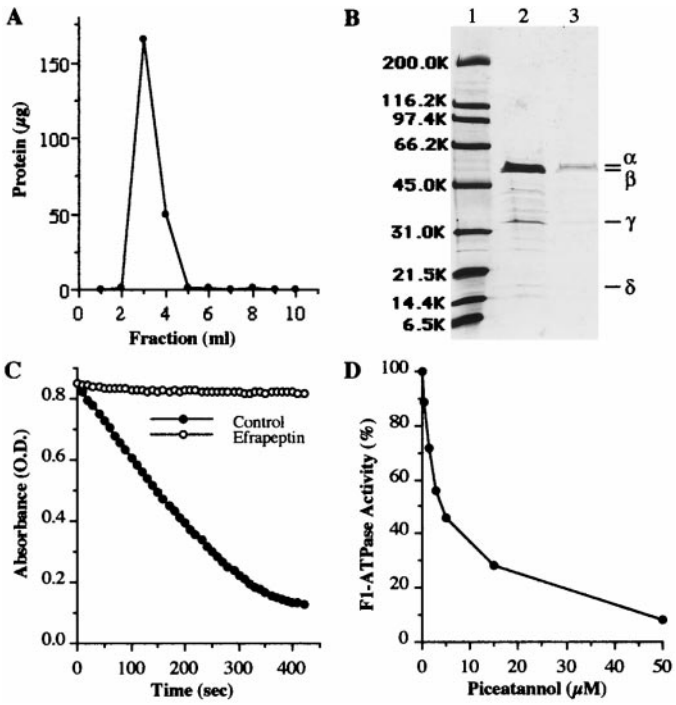

FIG. 3. Isolation of liver F1-ATPase and its inhibition by piceatannol. (A) F1-ATPase released after chloroform treatment was exchanged to stabilizing buffer using a PD-10 column and collected in 1 ml fractions. A small portion of each fraction was assayed for protein to obtain the total proteins in each fraction. (B) The protein-containing fractions 3 and 4 were examined by 4–20% SDS-PAGE. Lane 1, broad range protein standard markers. Lanes 2 and 3, 9 μl of original fractions 3 and 4 (about 1.3 μg and 0.38 μg protein, respectively). (C) The purified rat liver F1 (0.82 μg/0.7 ml) was highly active and sensitive to efrapeptin (1 μM). The control F1-ATPase specific activity was 19.6 μmol ATP hydrolyzed/min/mg protein. (D) Effect of piceatannol (0.5–50 μM) on liver F1-ATPase activity. The control F1-ATPase specific activity was 20.1 $\pm$ 0.5 μmol ATP hydrolyzed/min/mg protein (n = 3) and is considered as 100%.

several other compounds (Table 1). Resveratrol also inhibited the F1 with IC50 of slightly less than 15 μM (Table 1), a value similar to the inhibition detected on the F0F1-ATPase of solubilized brain and liver mitochondrial preparations (10). Consistent with earlier findings, quercetin, a diphenolic flavone, was also quite active (IC50 of about 3 μM). The potency of quercetin, however, is much higher in rat liver F1 than the F0F1-ATPase of solubilized rat brain mitochondrial preparation (IC50 of 50 μM, ref. 10), the bovine heart F1 (IC50 of 22 μM, ref. 21), or the porcine heart F1 (IC50 $>$ 41 μM, ref. 20). Genistein, on the other hand, was much less active on F1-ATPase (10% inhibition at 50 μM). Previously we have shown that genistein inhibited the F0F1-ATPase of solubilized brain mitochondrial preparation with an IC50 of 55 μM (10). Furthermore, both 17β-estradiol and 17α-estradiol, natural estrogens that inhibited the F0F1-ATPase activity of a solubilized brain mitochondrial preparation at μM concentrations (14), had little effect up to 50 μM. Therefore, genistein

TABLE 1

Effect of Selected Chemical Compounds on the Rat Liver F1-ATPase Activity Using an NADH-Linked ATP Regeneration System

Treatment	Concentration	Activity (%)
Control	—	100
Efrapeptin	1 μM	3.1 $\pm$ 1.9 (3)
Oligomycin	5 μg/ml	109.4 $\pm$ 1.6 (3)
Piceatannol	4 μM	50.1 $\pm$ 0.7 (3)
Resveratrol	15 μM	47.2 $\pm$ 2.8 (3)
Quercetin	1.5 μM	55.9
	5 μM	33.3
Genistein	50 μM	89.6 $\pm$ 4.0 (3)
17β-Estradiol	50 nM	102.9
	50 μM	100.3
17α-Estradiol	50 μM	96.3

Note. The control experiments were done in the presence of same amount of vehicle (water, ethanol or methanol). Mean values $\pm$ SD are indicated for some of the compounds. In parentheses is the number of trials.

and estradiol may preferentially target the F0 as shown earlier for diethylstilbestrol (22), while piceatannol, resveratrol, and quercetin are targeting the F1.

Effect of Piceatannol on Na^+, K^+-ATPase Activity

To examine the specificity of the effect of piceatannol, we have used a Na^+, K^+-ATPase preparation of porcine cerebral cortex. As shown in Fig. 4, piceatannol was relatively inactive on the Na^+, K^+-ATPase, since up to 7 μM, piceatannol had less than 2% inhibition. With increasing concentrations, piceatannol did inhibit the Na^+, K^+-ATPase activity. At 42 μM, piceatannol inhibited the ATPase activity by 30%.

Our studies therefore indicate that both piceatannol and resveratrol specifically target the F1 part of the F0F1-ATP synthase. Resveratrol is rich in red wine (common concentrations of 5–50 μM), grapes (skin and seeds), and peanut. It has been shown to bind to nuclear

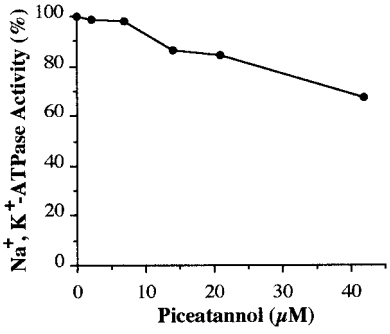

FIG. 4. Dose-dependent effect of piceatannol on the Na^+, K^+-ATPase activity of porcine cerebral cortex. The control activity is 0.168 $\pm$ 0.001 μmol ATP hydrolyzed/min/mg protein (n = 3) and is considered as 100%.

estrogen receptors, activate the transcription of estrogen-responsive reporter genes transfected into human breast cancer cells, and stimulate the proliferation of estrogen-dependent breast cancer cells at concentrations of 3–30 μM (23). It also stimulates the proliferation and differentiation of mouse osteoblastic MC3T3-E1 culture cells (24). However, *in vivo* experiments indicated that resveratrol did not act as an estrogen agonist in rats with doses of up to 1 mg/day (25). Our preliminary experiments indicate that picetannol at μM concentrations also inhibit the [^{3}H]estradiol binding to a rat uterine cytosol preparation rich in nuclear estrogen receptors with Ki similar to resveratrol (14 μM) (unpublished results). On the other hand, several experiments indicate that resveratrol inhibits the tumor cell growth *in vivo* (26, 27) and *in vitro* (28), apparently by an estrogen receptor-independent mechanism. The potential mechanisms of resveratrol's anti-tumor actions include the antioxidant action (29) and inhibition of cyclooxygenase (26). Piceatannol also inhibits the tumor cell growth, and one of the mechanisms could be due to its inhibitory effect on the PTK (4).

We demonstrated earlier that resveratrol and isoflavones (e.g., genistein) inhibited the mitochondrial F0F1-ATPase/ATP synthase (10), a ubiquitous enzyme that is responsible for synthesis of ATP, and therefore, critical for cell growth and survival. Here, we showed that piceatannol also inhibited this enzyme, more potent than resveratrol and isoflavones. The target site for both piceatannol and resveratrol is the F1 part of the F0F1-ATPase. Since the α and β subunits of the F1 have been localized on the plasma membranes of normal endothelial cells of blood vessels, and are target sites of angiostatin (13), an angiogenesis and tumor growth inhibitor, piceatannol and resveratrol could also act on the plasma membranes by binding to these subunits. On the other hand, genistein has only small effect on F1 (10% inhibition at 50 μM), while 17β-estradiol and 17α-estradiol are not effective up to 50 μM (Table 1). Therefore, these compounds may target the F0 part of the enzyme complex. A putative binding protein could be OSCP, a subunit of the F0F1-ATPase that is required for the coupling from proton gradient across the F0 to the ATP synthesis by the F1, and is a binding protein for [^{125}I]-labeled estradiol-bovine serum albumin (BSA) (30).

Our studies indicate that F0F1-ATPase/ATP synthase could be a potential target for stilbene phytochemicals from grape and several other medicinal plants. However, the contribution of this mechanism to the effects of these phytoalexins in human health is not yet clear and warrants further studies.

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