



# Inhibition of mitochondrial proton F<sub>0</sub>F<sub>1</sub>-ATPase/ATP synthase by polyphenolic phytochemicals

\*<sup>1</sup>Jianbiao Zheng & <sup>1,2,3</sup>Victor D. Ramirez

<sup>1</sup>Department of Molecular and Integrative Physiology, University of Illinois at Urbana-Champaign, Urbana, Illinois, IL 61801, U.S.A.; <sup>2</sup>Neuroscience Program, University of Illinois at Urbana-Champaign, Urbana, Illinois, IL 61801, U.S.A. and <sup>3</sup>College of Medicine, University of Illinois at Urbana-Champaign, Urbana, Illinois, IL 61801, U.S.A.

**1** Mitochondrial proton F<sub>0</sub>F<sub>1</sub>-ATPase/ATP synthase synthesizes ATP during oxidative phosphorylation. In this study, we examined the effects of several groups of polyphenolic phytochemicals on the activity of the enzyme.

**2** Resveratrol, a stilbene phytoalexin that is present in grapes and red wine, concentration-dependently inhibited the enzymatic activity of both rat brain and liver F<sub>0</sub>F<sub>1</sub>-ATPase/ATP synthase (IC<sub>50</sub> of 12–28 μM).

**3** Screening of other polyphenolic phytochemicals using rat brain F<sub>0</sub>F<sub>1</sub>-ATPase activity resulted in the following ranking potency (IC<sub>50</sub> in parenthesis): piceatannol (8 μM) > resveratrol (19 μM) = (–)epigallocatechin gallate (17 μM) > (–)epicatechin gallate, curcumin (45 μM) > genistein = biochanin A = quercetin = kaempferol = morin (55–65 μM) > phloretin = apigenin = daidzein (approx. 100 μM). Genistin, quercitrin, phloridzin, (+)catechin, (+)epicatechin, (–)epicatechin and (–)epigallocatechin had little effect at similar concentrations. Tannic acid, theaflavins (tea extract) and grape seed proanthocyanidin extract (GSPE) had IC<sub>50</sub> values of 5, 20 and 30 μg ml<sup>-1</sup>, respectively. Several monophenolic antioxidants and non-phenolic compounds were ineffective at concentrations of 210 μM or higher.

**4** The inhibition of F<sub>0</sub>F<sub>1</sub>-ATPase by resveratrol and genistein was non-competitive in nature.

**5** The effects of polyphenolic phytochemicals were additive.

**6** Both resveratrol and genistein had little effect on the Na<sup>+</sup>/K<sup>+</sup>-ATPase activity of porcine cerebral cortex, whereas quercetin had similar inhibitory potency as for F<sub>0</sub>F<sub>1</sub>-ATPase.

**7** In conclusion, the ATP synthase is a target for dietary phytochemicals. This pharmacological property of these phytochemicals should be included in the examination of their health benefits as well as potential cytotoxicity.

*British Journal of Pharmacology* (2000) **130**, 1115–1123

**Keywords:** Phytoestrogens; F<sub>0</sub>F<sub>1</sub>-ATPase/ATP synthase; Na<sup>+</sup>/K<sup>+</sup>-ATPase; flavonoids; mitochondria; resveratrol; genistein; catechins; tea

**Abbreviations:** AEBSF, 4-(2-aminoethyl)-benzenesulphonyl fluoride; CHAPS, (3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulphonate; c.i., confidence interval; ECG, (–)epicatechin gallate; EGCG, (–)epigallocatechin gallate; GSPE, grape seed proanthocyanidin extract; I3C, indole-3-carbinol; K<sub>m</sub>, Michaelis constant; OPC, oligomeric proanthocyanidin complex; OSCP, oligomycin sensitivity-conferring protein; P<sub>i</sub>, inorganic phosphate; V<sub>max</sub>, maximal specific activity

## Introduction

F<sub>0</sub>F<sub>1</sub>-ATPase/ATP synthase (F-type ATPase, complex V) is present in the inner membrane of eukaryotic mitochondria and acts as the powerhouse of the cell by synthesizing ATP. It can also operate in the reverse direction, hydrolysing ATP and pumping protons under certain conditions. The enzyme can be separated into two major complexes: F<sub>1</sub> and F<sub>0</sub> (Pedersen & Amzel, 1993; Boyer, 1997). F<sub>1</sub> is a water-soluble catalytic complex consisting of five subunits (α<sub>3</sub>β<sub>3</sub>γδε), with the catalytic site located on the β subunit. F<sub>0</sub> is made up of several membrane proteins (a, b, c, d, e, F<sub>6</sub>, A6L) and oligomycin sensitivity-conferring protein (OSCP), which contributes to the stalk region between F<sub>0</sub> and F<sub>1</sub>. In addition, a native peptide is also bound to the F<sub>1</sub> under de-energized conditions that serves to inhibit the ATPase activity of the enzyme (called F<sub>1</sub> inhibitor protein, IF<sub>1</sub>).

Recent studies suggest that both α and β subunits of the F<sub>1</sub>-ATPase may be present on the surface of human umbilical vein endothelial cells and are binding sites for angiostatin, a proteolytic fragment of plasminogen that is both a potent antagonist of angiogenesis and an inhibitor of tumour growth (Moser *et al.*, 1999). Antibodies against α subunit labelled the cell surface and inhibited angiostatin's anti-proliferative effect on endothelial cells. Earlier, β subunit had been localized to the cell surface of three human tumour cell lines (erythroleukaemia K562 cells, lung adenocarcinoma A549 cells and Burkitt's lymphoma Raji cells), but not to that of normal human erythrocytes or lymphocytes (Das *et al.*, 1994). The surface β subunit in tumour cells induces lymphocyte-mediated cell killing, whereas antibodies against the β subunit inhibit this cytotoxicity. These studies suggest the key involvement of cell surface F-ATPase subunits in tumour growth and inhibition, which could be potential therapeutic targets for cancers.

Several inhibitors of ATP synthase have been described, including efrapreptin, oligomycin, aurovertin B and azide (Linnett & Beechey, 1979). The binding pocket for the F<sub>1</sub>-

\*Author for correspondence at: Department of Molecular and Integrative Physiology, University of Illinois at Urbana-Champaign, 524 Burrill Hall, 407 South Goodwin Avenue, Urbana, IL 61801, U.S.A. E-mail: j-zheng@uiuc.edu

targeting inhibitor efrapeptin is localized in  $\alpha$ ,  $\beta$  and  $\gamma$  subunits (Abrahams *et al.*, 1996) while that for aurovertin B is localized mainly in the  $\beta$  subunit (van Raaij *et al.*, 1996). Inhibition of F0F1-ATPase/ATP synthase by the F0-targeting inhibitor, oligomycin, has been shown to protect or postpone cell injury by preserving ATP during ischaemia (Vuorinen *et al.*, 1995) and to induce apoptosis in tumour cells (Wolvetang *et al.*, 1994). Oligomycin also inhibits the apoptosis induced by Bax, a pro-apoptotic protein localized on the mitochondrial outer membrane, and F0F1-ATPase is required for the killing of cells by Bax (Matsuyama *et al.*, 1998).

We recently reported that rat brain F0F1-ATPase was retained by oestradiol affinity columns and that oestradiol and several other oestrogens inhibited its activity (Zheng & Ramirez, 1999a,b). Earlier, diethylstilbestrol, a synthetic oestrogen, was shown to inhibit rat liver F0F1-ATPase (McEnery & Pedersen, 1986; McEnery *et al.*, 1989). Although these effects require pharmacological (micromolar) concentrations of these oestrogens, which may not be physiologically relevant, phytoestrogens and other structurally similar polyphenolic phytochemicals are present in abundance in human food (Dewick, 1997; Bravo, 1998). It is worth mentioning that one group of weak oestrogenic phytochemicals—flavones, such as quercetin—has been shown to inhibit bovine and porcine heart F0F1-ATPase (Lang & Racker, 1974; Di Pietro *et al.*, 1975). Quercetin also inhibits a number of other ATPases, such as  $\text{Na}^+/\text{K}^+$ -ATPase,  $\text{Ca}^{2+}$ -ATPase and more than a dozen

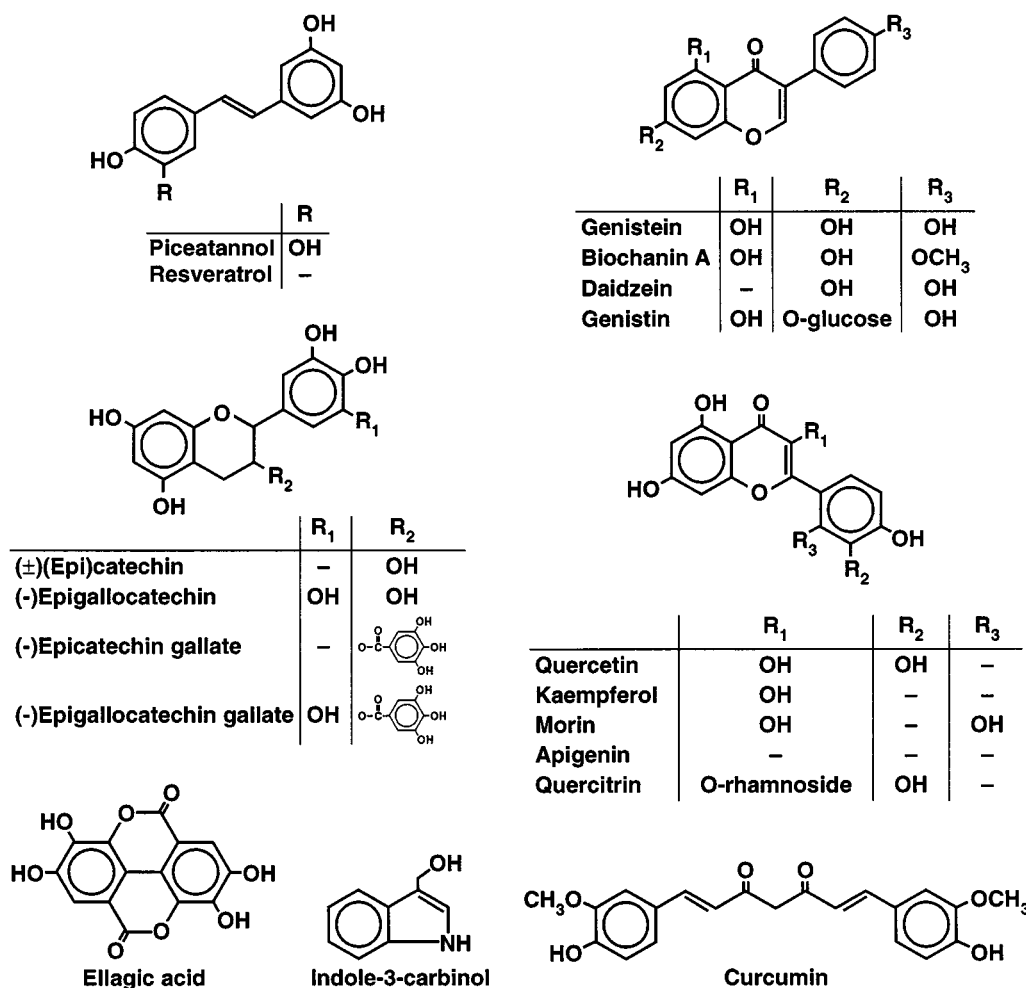
other enzymes (Fewtrell & Gomperts, 1977; Ferrell *et al.*, 1979; Hirano *et al.*, 1989; McKenna *et al.*, 1996).

In this study, we report on the effects of several groups of polyphenolic phytochemicals that have been shown to be important in human diets on the activity of mitochondrial F0F1-ATPase/ATP synthase, including stilbenes, isoflavones, flavones, catechins, chalcones and several others (Figure 1). In addition, we report the kinetic mechanisms of inhibition for selected compounds as well as their effect on  $\text{Na}^+/\text{K}^+$ -ATPase. Many of these phytochemicals exhibit diverse activities such as antioxidant, cardiovascular protective, anti-osteoporotic, cancer chemopreventative, anti-mitotic and oestrogenic actions (Frankel *et al.*, 1993; Adlercreutz, 1995; Gehm *et al.*, 1997; Jang *et al.*, 1997; Hsieh *et al.*, 1998; Dashwood, 1998; Setchell, 1998; Murkies *et al.*, 1998; Tham *et al.*, 1998; Hollman *et al.*, 1999; Yang *et al.*, 1999; Ye *et al.*, 1999). Our study, therefore, could provide a potential mechanism for the actions of phytochemicals through targeting of the F0F1-ATPase/ATP synthase.

## Methods

### Materials

4-(2-Aminoethyl)-benzenesulphonyl fluoride (AEBSF), an irreversible serine protease inhibitor, was from Boehringer Mannheim (Indianapolis, IN, U.S.A.). Oligomycin (mixture of



**Figure 1** Structures of selected phytochemicals tested for their inhibitory effects on the rat mitochondrial F0F1-ATPase/ATP synthase activity. Many have been shown to be phytoestrogens, such as stilbenes, isoflavones and flavones.

A, B and C) was purchased from Aldrich (Milwaukee, WI, U.S.A.) and prepared in methanol as a stock solution ( $1 \mu\text{g} \mu\text{l}^{-1}$ ). Efrapeptin (efrastatin, A23871) was kindly provided by Dr J. Clemens (Eli Lilly & Co., Indianapolis, IN, U.S.A.) and dissolved in sterile distilled water at a concentration of 0.1 mM. IH636 Grape seed proanthocyanidin (GSPE) was kindly provided by Dr. D. Bagchi (InterHealth Nutraceuticals, Concord, CA, U.S.A.) as a mixture of proanthocyanidins (>54% dimeric, 13% trimeric, 6.8% tetrameric and small amount of monomeric and other high molecular weight oligomeric proanthocyanidin complexes [OPC]). All other chemicals and reagents, including porcine cortex  $\text{Na}^+/\text{K}^+$ -ATPase were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Ouabain (stock solution of  $5 \mu\text{g} \mu\text{l}^{-1}$ ) was dissolved in sterile distilled water. Phytochemicals and diethylstilbestrol were usually prepared in 100% ethanol with a final concentration of 10 mM or above, except for daidzein, phloridzin and GSPE, which were prepared in 50% ethanol/50% dimethyl sulphoxide. The final solvent concentrations in the reaction solution were usually 0.7% and occasionally 1.4%. The amount of solvents had minimal effect on the responses (<5%). Nevertheless, in every experiment, control trials were performed with the same amount of vehicle included.

### Animals

Adult female Sprague-Dawley rats (60–120 days old) were maintained on a 14:10 h light/dark cycle (lights on at 0700 h) with food and water available *ad libitum*. Animals were taken care of in accordance with federal and institutional guidelines and killed by rapid decapitation.

### Preparation of mitochondria

Mitochondria fractions from whole brain and liver of adult Sprague-Dawley female rats (unknown oestrous cycle) were prepared in Tris buffer (mM: Tris-HCl 50, NaCl 120, KCl 5,  $\text{MgSO}_4$  1,  $\text{CaCl}_2$  1, 10% glycerol, AEBSF 0.5, bacitracin 0.1; pH 7.4 at  $4^\circ\text{C}$ ), as described previously (Zheng & Ramirez, 1999a). Briefly, tissues were homogenized in Tris buffer (10 ml per g tissue) with a Teflon glass homogenizer (10 strokes in 1 min). Homogenates were centrifuged at  $600 \times g$  for 10 min and the resulting supernatant was centrifuged again at  $15,000 \times g$  for 5 min to precipitate the mitochondrial fraction. Samples were assayed for protein concentration by the method of Bradford (1976), using bovine serum albumin as the standard, and either kept at  $4^\circ\text{C}$  for use the same day or stored at  $-80^\circ\text{C}$ .

### Solubilization

Mitochondrial fractions were solubilized in Tris buffer containing 1% w  $v^{-1}$  digitonin (Zheng & Ramirez, 1999a) or 1% 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propane sulphate (CHAPS) at  $4^\circ\text{C}$  as described (McEnery *et al.*, 1984). Solubilization allows the exposure of F<sub>0</sub>F<sub>1</sub>-ATPase, which is not accessible to many reagents in a coupled assay (see below), in intact mitochondria.

### Preparation of submitochondrial particles

Submitochondrial particles were prepared by sonication as described (Ragan *et al.*, 1987). Briefly, freshly prepared brain mitochondrial fractions were sonicated for  $6 \times 10$  s and centrifuged at  $15,000 \times g$  for 5 min at  $4^\circ\text{C}$ . The supernatant

was centrifuged again at  $125,000 \times g$  for 60 min; the resulting pellet (submitochondrial particles) was resuspended in Tris buffer and used within 24 h.

### Assay for ATPase activity

The mitochondrial F<sub>0</sub>F<sub>1</sub>-ATPase activity was measured spectrophotometrically at 340 nm by coupling the production of ADP to the oxidation of NADPH *via* the pyruvate kinase and lactate dehydrogenase reaction (coupled assay), as described (Zheng & Ramirez, 1999b). The reaction mixture (0.7 ml final volume) contained (in mM): Tris 100 (pH 8.0), Mg-ATP 4,  $\text{MgCl}_2$  2, KCl 50, EDTA 0.2, NADH 0.23, phosphoenol pyruvate 1, 1.4 unit pyruvate kinase, 1.4 unit lactate dehydrogenase and about 25–50  $\mu\text{g}$  proteins, and was assayed at 30–31 $^\circ\text{C}$ . The activity of the  $\text{Na}^+/\text{K}^+$ -ATPase from porcine cerebral cortex was measured similarly by the coupled assay as described above except that 100 mM NaCl was included. In a few cases, the F<sub>0</sub>F<sub>1</sub>-ATPase activity was also measured directly from the release of inorganic phosphate ( $\text{P}_i$ ) from ATP as described (Harris, 1987). In this case the reaction solution (0.7 ml) contained (in mM): Tris 100, (pH 8.0), Mg-ATP 4,  $\text{MgCl}_2$  2, KCl 50, EDTA 0.2 and 25–50  $\mu\text{g}$  proteins. The specific F<sub>0</sub>F<sub>1</sub>-ATPase activity in all cases was determined in the presence of the enzyme inhibitors, oligomycin or efrapeptin (Linnett & Beechey, 1979). Ouabain was used to determine the specific  $\text{Na}^+/\text{K}^+$ -ATPase activity.

To study possible effects of phytochemicals on the other enzymes used in the coupled assay of ATPase, *i.e.* pyruvate kinase and lactate dehydrogenase, ATP was omitted from the buffer and the reaction was started by adding 0.2 mM ADP.

### Assay for ATP synthesis

ATP synthesis was measured by monitoring the increase in absorbance at 340 nm using an  $\text{NADP}^+$ -linked, ADP-regenerating system (Cross & Kohlbrenner, 1978). The reaction mixture (0.7 ml final volume) contained (mM): HEPES 10 (pH 8.0), succinate 20, glucose 20,  $\text{MgCl}_2$  3, AMP 11,  $\text{NADP}^+$  0.75, ADP 1,  $\text{P}_i$  10, 4 u  $\text{ml}^{-1}$  hexokinase and 2 u  $\text{ml}^{-1}$  glucose-6-phosphate dehydrogenase. The reaction was assayed at 30–31 $^\circ\text{C}$  and started by adding 20  $\mu\text{l}$  of pre-incubated (5 min at  $26^\circ\text{C}$ ) submitochondrial particles (about 50–61  $\mu\text{g}$  protein) in 20 mM succinate. A high AMP concentration (11 mM) was used in this assay to inhibit the adenyl kinase activity present in the submitochondrial particle preparations, which might interfere with measurement of respiration-dependent ATP synthesis by the ATP synthase. The preparations had ATP synthase activity of 0.057–0.083  $\mu\text{mol min}^{-1}$  ( $\text{mg protein}^{-1}$ ).

To exclude the possible effects of resveratrol on hexokinase and glucose-6-phosphate dehydrogenase used in the assay, the response induced by 140  $\mu\text{M}$  ATP in the absence of submitochondrial particles were tested; these were not significantly affected.

### Data analysis

To obtain reaction rates, the steady-state linear range of the absorbance change with respect to time was used for both ATPase and ATP synthase activity. A molar extinction coefficient of  $6.22 \times 10^3 \text{ M cm}^{-1}$  was used for NAD(P)H to calculate the activity in  $\mu\text{mol ATP}$  hydrolysed or synthesized per min per mg protein. The responses were very stable as judged from control trials performed before, during and after the experiments (less than 4% variations). Data from three or

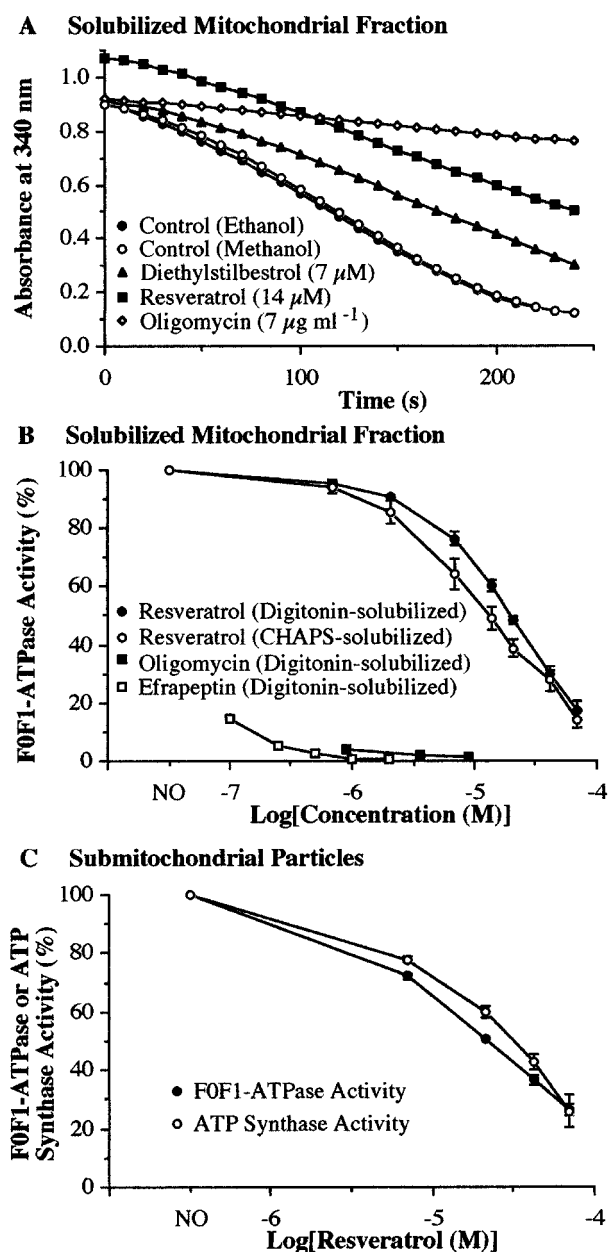
more experiments were expressed as means (standard deviation (s.d.)). Statistical analysis was performed using Student's *t*-test to compare two groups and analysis of variance (ANOVA) with *post-hoc* Tukey test for comparison of three or more groups.  $P < 0.05$  was considered significant. For concentration-dependent effects of resveratrol with an experimental number of 3,  $IC_{50}$  values are quoted as geometric mean with 95% confidence interval (c.i., based on *t* distribution).

## Results

### *Inhibition of rat F0F1-ATPase/ATP synthase by resveratrol*

Mitochondrial fractions prepared from both female rat brains and livers were solubilized with 1% digitonin or 1% CHAPS to obtain functional solubilized mitochondrial fractions containing high F0F1-ATPase activity with little  $Na^+/K^+$ -ATPase activity in a coupled ATPase activity assay (Zheng & Ramirez, 1999b). Resveratrol, a phytoalexin present in high concentrations in red wine (usually 10–50  $\mu M$ ) and grapes, rapidly (within 1–2 min) inhibited the brain mitochondrial F0F1-ATPase activity in a concentration-dependent fashion in the range 0.7–70  $\mu M$ , with  $IC_{50}$  values of 18.5  $\mu M$  (geometric mean, 95% c.i. 16.7–20.6  $\mu M$ ;  $n=3$ ) and 13.0  $\mu M$  (8.80–19.3  $\mu M$ ;  $n=3$ ) for digitonin-solubilized and CHAPS-solubilized preparations, respectively (Figure 2A,B). With 0.7  $\mu M$  of resveratrol, the inhibition was already significant (5–7% inhibition,  $P < 0.01$ ). Similar results were obtained with both digitonin- and CHAPS-solubilized liver mitochondrial preparations ( $IC_{50}$  of 12 and 21  $\mu M$ , respectively). The inhibitory effect of resveratrol appears to be specific to F0F1-ATPase given that this effect is blocked by oligomycin, an F0F1-ATPase/ATP synthase inhibitor (Linnett & Beechey, 1979). In the presence of 7  $\mu g\ ml^{-1}$  oligomycin alone, the residual ATP hydrolysis activity in the digitonin-solubilized preparation was 16.1 (0.3)% (mean(s.d.);  $n=3$ ) when the control activity was assumed to be 100%. In the presence of both 7  $\mu g\ ml^{-1}$  oligomycin and 21  $\mu M$  resveratrol, the residual activity was 15.8 (0.4)% ( $n=3$ ). Efrapeptin, a specific inhibitor of the F0F1-ATPase/ATP synthase (Linnett & Beechey, 1979), completely inhibited the enzyme activity at 1–2  $\mu M$  (Figure 2B). Therefore, the effect of resveratrol must be on the oligomycin and efrapeptin-sensitive ATPase (i.e. F0F1-ATPase) activity of this preparation, which corresponded to an average of 83.5 (2.2)% ( $n=7$ ) of the total ATP hydrolysis activity. Resveratrol itself did not have any effect on the activity of the other enzymes, i.e. pyruvate kinase and lactate dehydrogenase, used in the ATPase activity assay because, in the absence of mitochondrial preparations, 21  $\mu M$  resveratrol did not affect the ADP-induced NADH oxidation. In the presence of 21  $\mu M$  resveratrol, the ADP-induced response was 100.2 (1.0)% ( $n=3$ ) of control.

To examine the effects of resveratrol on ATP synthesis by the F0F1-ATPase/ATP synthase we used a brain submitochondrial fraction. In the presence of 11 mM AMP the brain submitochondrial particles contained little adenylate kinase activity: more than 95% of the activity corresponded to ATP synthase as shown in the presence of 2  $\mu M$  efrapeptin (data not shown). Resveratrol inhibited ATP synthesis with an  $IC_{50}$  of 27.7  $\mu M$  (22.0–34.8  $\mu M$ ; Figure 2C) and had no effect on the other enzymes used in the assay. Resveratrol also inhibited the F0F1-ATPase activity of the submitochondrial particles with an  $IC_{50}$  of 21.6  $\mu M$  (20.6–22.8  $\mu M$ ; Figure 2C). Therefore, it seems that resveratrol inhibited both ATP synthesis and



**Figure 2** Effects of resveratrol on F0F1-ATPase/ATP synthase activity of rat brain mitochondrial preparations. (A) Typical examples of spectrophotometric read-out showing the absorbance change at 340 nm induced by 20  $\mu l$  digitonin-solubilized brain mitochondrial preparation (51  $\mu g$  protein) in the presence of ethanol vehicle, methanol vehicle, 7  $\mu g\ ml^{-1}$  oligomycin in methanol, 7  $\mu M$  diethylstilbestrol in ethanol and 14  $\mu M$  resveratrol in ethanol. Resveratrol itself and, to a lesser degree, diethylstilbestrol and oligomycin, had extinction at 340 nm, so the starting point had higher absorbance. Diethylstilbestrol was included here as a comparison. Oligomycin and efrapeptin (see below and text) were included for determination of the F0F1-ATPase activity in the preparations. (B) The concentration-dependent effect of 0.7–70  $\mu M$  resveratrol on digitonin- and CHAPS-solubilized rat brain mitochondrial preparations. Data are expressed as means (s.d.) from three experiments. Also shown is the concentration-dependent effect of oligomycin and efrapeptin. For studying the effect of efrapeptin on ATPase activity, efrapeptin was pre-incubated at 26°C with the solubilized preparation for 6 min to allow for binding. The F0F1-ATPase activity in 0.7% vehicle (this and following figures) was defined as 100%. (C) Effect of resveratrol on ATP synthesis and hydrolysis catalysed by rat brain submitochondrial preparations. F0F1-ATPase activity of rat brain submitochondrial preparations was determined similarly as solubilized mitochondrial preparation except that 2  $\mu g\ ml^{-1}$  antimycin A was present. Data are expressed as means (s.d.) from three experiments. The F0F1-ATPase/ATP synthase activity in vehicle was defined as 100%.

hydrolysis by the enzyme, but the effect on the ATP synthase activity is slightly less than on the ATPase activity.

Direct measurement of  $P_i$  release from ATP catalysed by digitonin-solubilized brain mitochondrial preparation indicated that  $70 \mu\text{M}$  resveratrol inhibited the oligomycin and efrapreptin-sensitive F0F1-ATPase activity by 84.6%, a value similar to that determined by the coupled assay.

#### Differential inhibition of F0F1-ATPase by polyphenolic phytochemicals

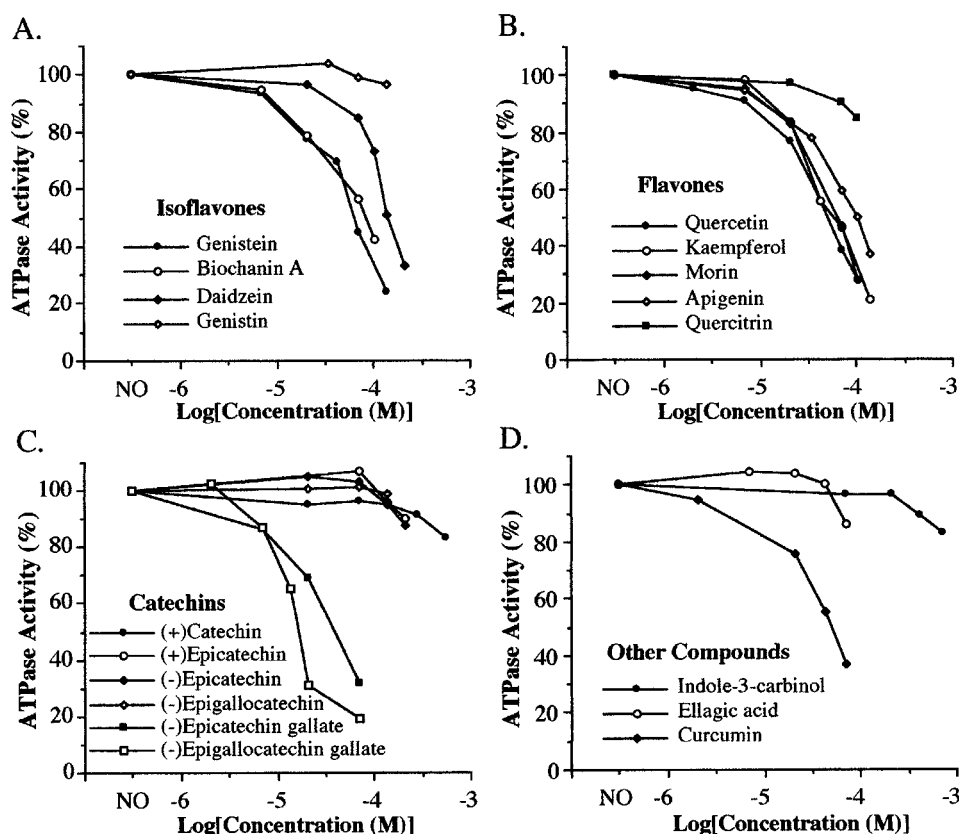
We further screened other phenolic phytochemicals for their effects on the activity of the ATPase in digitonin-solubilized rat brain mitochondrial preparation (Figure 3, Table 1). Three isoflavones, genistein, biochanin A and daidzein—phytoalexins with protein anti-tumour effects—inhibited the ATPase activity with  $IC_{50}$  values of 55–127  $\mu\text{M}$  (Figure 3A, Table 1). However, the 7-glucose derivative of genistein (genistin) was essentially without effect at concentrations up to 140  $\mu\text{M}$ . The isoflavone compounds had little effect on ADP-induced responses in the absence of the mitochondrial preparations (<10% effect at 70  $\mu\text{M}$ ) and, therefore, their actions are due to effects on the ATPase. Genistein and biochanin A, like resveratrol, did not significantly affect the oligomycin-insensitive ATP hydrolysis activity. In the presence of 7  $\mu\text{g ml}^{-1}$  oligomycin, 15.3% residual ATP hydrolysis activity remained. When 70  $\mu\text{M}$  genistein or biochanin A was added in the presence of 7  $\mu\text{g ml}^{-1}$  oligomycin, the residual ATP hydrolysis activity

was 13.4 and 12.9%, respectively. We also tested the effects of genistein on ATP synthase activity and found that at 70  $\mu\text{M}$  it also inhibited the enzymatic activity by 62%. Therefore, like resveratrol, genistein inhibits both ATPase and ATP synthase activity of the enzyme.

Consistent with earlier findings (Lang & Racker, 1974; Di Pietro *et al.*, 1975), aglycone flavones also inhibited the brain F0F1-ATPase activity with  $IC_{50}$  values of 50–105  $\mu\text{M}$ , while quercitrin had little effect (Figure 3B). When several catechins were tested it was found that diphenolic catechin, epicatechin and epigallocatechin had little effect on the F0F1-ATPase activity, while the gallate esters, including (–)epigallocatechin gallate (EGCG) and (–)epicatechin gallate (ECG) are potent inhibitors (Figure 3C). Other active inhibitors of F0F1-ATPase include curcumin (Figure 3D), phloretin, tea theaflavins, tannic acid and GSPE (Table 1). Ellagic acid had a modest inhibitory effect on F0F1-ATPase activity (14% at 70  $\mu\text{M}$ ) (Figure 3D). Monophenolic compounds, such as caffeic acid, gallic acid and salicylic acid, and nonphenolic compounds, such as indole-3-carbinol (I3C) and tartaric acid, had little effect at similar concentrations (Table 1).

#### Kinetic mechanism of inhibition by resveratrol and genistein

We further examined the effects of resveratrol and genistein on the ATP-dependence of the inhibition of F0F1-ATPase. As shown in Figure 4, the control Lineweaver-Burk plot revealed

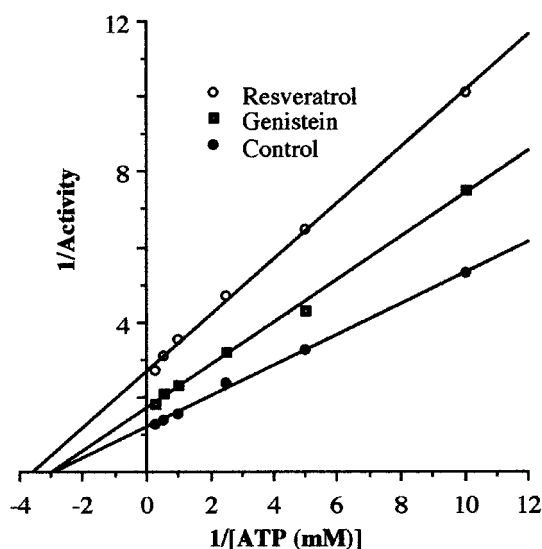


**Figure 3** Concentration-dependent effects of selected phytochemicals on total ATPase activity of digitonin-solubilized rat brain mitochondrial preparations. These compounds include isoflavones (A), flavones (B), catechins (C) and several others (D). Experiments were conducted as in Figure 2. Control experiments indicated that (–)epicatechin-gallate, tannic acid and several flavonoids affected ADP-induced responses, i.e. pyruvate kinase or lactate dehydrogenase; therefore, the total ATPase activity was used here. The inhibition of ADP-induced responses was from 28 to 76% at 70  $\mu\text{M}$  with kaempferol < apigenin < quercetin < (–)epicatechin gallate < morin. This inhibition, however, will not significantly affect the estimation of  $IC_{50}$  for the ATPase since ATP hydrolysis was the limiting step and the residual oligomycin-insensitive ATPase activity is low (13.4 (1.1)% of total activity). ‘NO’ indicates the absence of phytochemicals.

**Table 1** Effect of phytochemicals on the mitochondrial F<sub>0</sub>F<sub>1</sub>-ATPase activity

Compound	IC <sub>50</sub> (μM)	Compound	IC <sub>50</sub> (μM)
<b>Stilbenes</b>		<b>Chalcones</b>	
Piceatannol	8	Phloretin	40% at 70 μM
Resveratrol	19	Phloridzin	<15% up to 70 μM
<b>Isoflavones</b>		<b>Other polyphenolics</b>	
Genistein	55	Tannic acid	5 μg ml <sup>-1</sup>
Biochanin A	65	Theaflavins	20 μg ml <sup>-1</sup>
Daidzein	127	GSPE	30 μg ml <sup>-1</sup>
Genistin	<5% up to 140 μM	Curcumin	40
<b>Flavones</b>		<b>Ellagic acid</b>	
Quercetin	50		<15% up to 70 μM
Kaempferol	55	<b>Monophenolics</b>	
Morin	60	Caffeic acid	<15% up to 280 μM
Apigenin	105	Gallic acid	<15% up to 280 μM
Quercitrin	<15% up to 105 μM	Salicylic acid	<5% up to 700 μM
<b>Catechins</b>		<b>Non-phenolics</b>	
EGCG	17	Indole-3-carbinol	<15% up to 420 μM
ECG	45	Tartaric acid	<5% up to 7000 μM
(-)Epigallocatechin	<5% up to 140 μM		
(-)Epicatechin	<15% up to 210 μM		
(+)Epicatechin	<15% up to 210 μM		
(+)Catechin	<15% up to 280 μM		

The F<sub>0</sub>F<sub>1</sub>-ATPase activity of digitonin-solubilized rat brain mitochondrial preparations was determined using an NADH-linked ATP regeneration system. Control experiments were performed in the presence of same amount of vehicle and the variation between control trials was less than 4%. The effect of piceatannol (Zheng & Ramirez, 1999c) and several flavones (Lang & Racker, 1974; Di Pietro *et al.*, 1975) have been shown previously.



**Figure 4** Inhibition of F<sub>0</sub>F<sub>1</sub>-ATPase of digitonin-solubilized brain mitochondrial preparation by resveratrol (7 μM) and genistein (50 μM) at several concentrations of 0.1–4 mM ATP as shown by Lineweaver–Burk plots. The linear correlation coefficients for all three groups are between 0.996 and 0.999. Note the different  $V_{\max}$  among the control, resveratrol and genistein, and the change in  $K_m$  induced by resveratrol. The control F<sub>0</sub>F<sub>1</sub>-ATPase activity of the preparation at 4 mM ATP was 0.797 (0.007) μmol ATP hydrolysed min<sup>-1</sup> (mg protein)<sup>-1</sup> from three trials performed before, during and after the experimental trials.

maximal specific activity ( $V_{\max}$ ) for this preparation of 0.834 μmol ATP hydrolysed min<sup>-1</sup> (mg protein)<sup>-1</sup> with Michaelis constant ( $K_m$ ) of 0.344 mM. In the presence of resveratrol (7 μM),  $V_{\max}$  was drastically reduced to 0.370 μmol ATP hydrolysed min<sup>-1</sup> (mg protein)<sup>-1</sup>.  $K_m$  was also reduced by 20% (0.277 mM) in the presence of 7 μM resveratrol, suggesting that resveratrol results in mixed inhibition of F<sub>0</sub>F<sub>1</sub>-

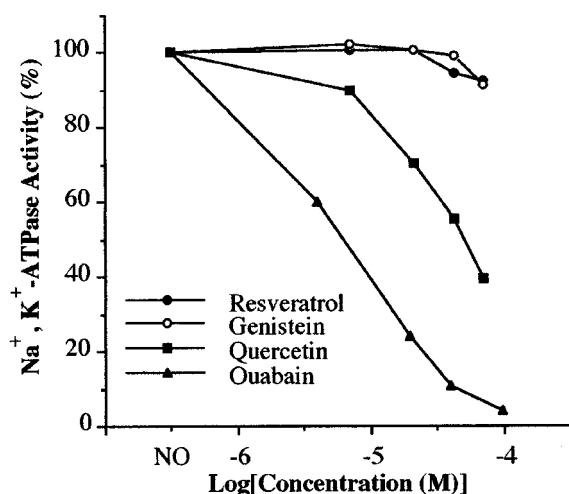
ATPase. Genistein (50 μM) also reduced the  $V_{\max}$  to 0.580 μmol min<sup>-1</sup> (mg protein)<sup>-1</sup>, while  $K_m$  was relatively unchanged (0.331 mM; 3.8% decrease), suggesting that genistein behaves like a classical non-competitive inhibitor.

#### Additive effect of polyphenolic compounds

To determine if resveratrol and other phytochemicals additively contribute to the inhibitory effect of dietary phytochemicals on F<sub>0</sub>F<sub>1</sub>-ATPase, we examined the effect of a combination of resveratrol with other active inhibitors at suboptimal concentrations. A combination of suboptimal concentrations of resveratrol, quercetin and kaempferol, which are present in red wine, had an additive inhibitory effect resulting in 56% inhibition as compared to single compounds alone (23, 19 and 17% inhibition for 7 μM resveratrol, 21 μM quercetin and 21 μM kaempferol, respectively) ( $P < 0.001$ ). Similar additive effects were found for resveratrol and genistein, two compounds that are usually not present in the same diet; the combination of 11 μM resveratrol and 35 μM genistein resulted in 58% inhibition as compared to 36% with resveratrol and 26% with genistein alone ( $P < 0.001$ ). Therefore, the presence of these or other active compounds in diet could potentially act together to alter the enzymatic activity of the F<sub>0</sub>F<sub>1</sub>-ATPase *in vivo* with considerably lower concentrations of individual compounds.

#### Lack of effect of resveratrol and genistein on Na<sup>+</sup>/K<sup>+</sup>-ATPase

To determine if resveratrol and genistein inhibit the activity of other ATPases, we examined their effect on Na<sup>+</sup>/K<sup>+</sup>-ATPase from porcine cerebral cortex. Resveratrol and genistein had little effect on this enzyme's activity (Figure 5). Therefore, the stilbene resveratrol and the isoflavone genistein are rather specific in their actions on mitochondrial ATPase/ATP synthase, unlike flavones such as quercetin, which were shown



**Figure 5** Effects of resveratrol, genistein and quercetin on porcine cerebral cortex  $\text{Na}^+/\text{K}^+$ -ATPase. Each point corresponds to a single trial except for control experiments, which were conducted at least three times with variation less than 3%.

to affect many ATPases, including  $\text{Na}^+/\text{K}^+$ -ATPase (Figure 5) and several other enzymes such as 5'-nucleotidase, phosphor-ylase kinase and protein kinase C, while genistein did not (Akiyama *et al.*, 1987).

## Discussion

By using both rat brain and liver mitochondrial preparations, we have tested several groups of naturally occurring polyphenolic compounds on the activity of rat mitochondrial F<sub>0</sub>F<sub>1</sub>-ATPase/ATP synthase. Our studies demonstrate that F<sub>0</sub>F<sub>1</sub>-ATPase/ATP synthase is a common target site for resveratrol from red wine, aglycone isoflavones (genistein, biochanin A and daidzein) from soybean, gallate esters of catechins from many sources, and several other polyphenolic compounds at high nanomolar to low micromolar concentrations similar to those used in many biological studies (e.g. Barnes & Peterson, 1995; Jang *et al.*, 1997; Yang *et al.*, 1999). The effect of resveratrol is consistent with recent demonstration of its inhibitory effect on the ATPase activity of intact rat brain mitochondria (Zini *et al.*, 1999). Our recent studies indicate that resveratrol, like piceatannol and quercetin, inhibits the F<sub>0</sub>F<sub>1</sub>-ATPase activity by targeting F<sub>1</sub>, while genistein most likely targets F<sub>0</sub> (Zheng & Ramirez, 1999c). Therefore, there are two potential binding sites in ATP synthase for polyphenolic phytochemicals. Interaction of phytochemicals with these binding sites could be a novel mechanism for the actions of these phytochemicals.

Comparison of several groups of polyphenolic phytochemicals indicate the importance of hydroxyl groups in particular positions. For example, three isoflavones – genistein, biochanin A and daidzein – inhibited the ATPase activity with  $\text{IC}_{50}$  values between 55 and 127  $\mu\text{M}$ , while the 7-glucose derivative of genistein (genistin) was essentially without effect up to 140  $\mu\text{M}$ . This, together with the fact that all three active isoflavones contain a 7-OH group, suggests that the 7-OH group is essential for the inhibitory effect of isoflavones. Omission of the 5-OH group in daidzein ( $\text{IC}_{50}$  = 127  $\mu\text{M}$ ) resulted in lower activity in comparison with genistein ( $\text{IC}_{50}$  = 55  $\mu\text{M}$ ). However, modification of the 4'-OH group to 4'-OCH<sub>3</sub> in biochanin A ( $\text{IC}_{50}$  = 65  $\mu\text{M}$ ) seems to have relatively little effect. It seems that a *meta*-quinonic structure could be essential for the

inhibitory activity. Similarly to genistin, the replacement of quercetin's R1-OH group by *O*-rhamnoside in quercitrin also resulted in much lower inhibitory activity. On the other hand, addition of a phenolic group in catechins converted inactive catechins to active catechins (the gallate esters, EGCG and ECG). The lack of inhibitory activity of monophenolic compounds tested suggests that the inhibition of F<sub>0</sub>F<sub>1</sub>-ATPase by phenolic phytochemicals requires two or more phenolic structures.

The levels of several phytochemicals in human body fluids have been determined. For instance, both genistein and daidzein (both free and conjugated forms) reached up to 7  $\mu\text{M}$  in human plasma after soy meals (Xu *et al.*, 1995; Watanabe *et al.*, 1998; King & Bursill, 1998). Administration of decaffeinated green tea could result in maximal plasma concentrations of 0.7 and 1.8  $\mu\text{M}$  in human volunteers for EGCG and EGC, respectively (Yang *et al.*, 1999). However, the levels of these compounds in saliva could reach 10–48 and 38–143  $\mu\text{M}$ , respectively (Yang *et al.*, 1999). These data indicate that much higher concentrations of phytochemicals are present in the oral cavity, and likely in the oesophagus, and that they could be important for the application of tea in the prevention of oral and oesophageal cancers (Yang *et al.*, 1999). Many of these polyphenolic phytochemicals are present in the same plant or food source or may be taken together. For example, red wine contains resveratrol, quercetin, kaempferol and catechins. Therefore, it is important to know that the effects of these compounds on ATPase are additive. This may explain why several brands of red wine and red grape juice, but not white wine, beer or ethanol, inhibit the ATPase activity with  $\text{IC}_{50}$  values of 0.7–1.4%  $v/v^{-1}$  (unpublished data). The lack of effect of white wine on F<sub>0</sub>F<sub>1</sub>-ATPase activity is consistent with several earlier studies showing that red wine and grape juice, but not white wine, have cardioprotective and tumour-inhibiting actions in animal models (Demrow *et al.*, 1995) and humans (Furhman *et al.*, 1995; Serafini *et al.*, 1998). Total polyphenolics, including resveratrol and proanthocyanidins, are several-fold lower in white wine than in red wine (Goldberg *et al.*, 1995; Watkins, 1997) which may explain different effects of red and white wine.

Many of these polyphenolic phytochemicals are weak oestrogens (phytoestrogens). For example, resveratrol is a weaker oestrogen receptor  $\alpha$  form agonist, having an effect only when its concentrations are above 3  $\mu\text{M}$  (3–30  $\mu\text{M}$  with  $\text{IC}_{50}$  = 10  $\mu\text{M}$ ) (Gehm *et al.*, 1997). The aglycone isoflavones are more potent phytoestrogens than resveratrol, with their affinity for oestrogen receptor  $\alpha$  and  $\beta$  forms in the following rank order: genistein > daidzein > biochanin A (Kuiper *et al.*, 1998). While biochanin A had a similar inhibitory effect as genistein on F<sub>0</sub>F<sub>1</sub>-ATPase activity (Table 1) and tumour growth (Barnes & Peterson, 1995), it has 400 to 8700 fold lower affinity than genistein for oestrogen receptor  $\alpha$  and  $\beta$  forms (Kuiper *et al.*, 1998). Therefore, it is likely that oestrogen receptors are not involved in anti-growth actions of these phytochemicals on cultured cancer cells and that the structure-activity relationship for F<sub>0</sub>F<sub>1</sub>-ATPase/ATP synthase inhibition by these phytochemicals is different from that for affinity at oestrogen receptors.

Besides its oestrogen receptor-mediated actions, genistein also inhibits protein tyrosine kinases, while biochanin A and daidzein are at least 40 times less active than genistein (Akiyama *et al.*, 1987). However, several earlier studies indicated that the tyrosine kinase was not required for the inhibitory effect of genistein on tumour growth (Barnes & Peterson, 1995; Stevens *et al.*, 1994; Shao *et al.*, 1998), nor for biochanin A and daidzein. Other mechanisms have been

suggested, including the inhibition of cyclo-oxygenase-1 (Jang *et al.*, 1997) and cytochrome P450 1A1 (Ciolino & Yeh, 1999; Chun *et al.*, 1999) by resveratrol, and inhibition of 5 $\alpha$ -reductase by genistein and biochanin A (Evans *et al.*, 1995). However, the involvement of these and other mechanisms in both cardioprotection and tumour inhibition by diphenolic phytochemicals is unclear.

Several flavones have been shown to interact with other ATPases, such as Ca<sup>2+</sup>-ATPase (McKenna *et al.*, 1996) and Na<sup>+</sup>/K<sup>+</sup>-ATPase (Hirano *et al.*, 1989), in addition to their effects on F0F1-ATPase (Lang & Racker, 1974; Di Pietro *et al.*, 1975). However, resveratrol and genistein had little effect on Na<sup>+</sup>/K<sup>+</sup>-ATPase, as shown in Figure 5. On the other hand, ellagic acid has been shown to increase the activity of Ca<sup>2+</sup>-ATPase and Ca<sup>2+</sup> uptake in cardiac sarcoplasmic reticulum (EC<sub>50</sub> = 10  $\mu$ M) (Antipenko *et al.*, 1999). However, ellagic acid has little effect on F0F1-ATPase of rat brain solubilized mitochondrial preparation at similar concentrations.

Our findings indicate that inhibition of mitochondrial F0F1-ATPase/ATP synthase could be a potential mechanism contributing to the many effects of dietary polyphenolics. It has been shown that the same ATP synthase is localized also on the plasma membranes of several tumour cell lines (Das *et al.*, 1994) and human umbilical vein endothelial cells, and that it is a binding protein for angiostatin, a potent protein inhibitor of angiogenesis and cell proliferation (Moser *et al.*, 1999). Therefore, phytoestrogens could also play a similar role

by binding the ATP synthase on the plasma membrane. In fact, genistein has been shown to be a potent inhibitor of angiogenesis (Fotsis *et al.*, 1993). Moreover, resveratrol and genistein also inhibit the bovine mitochondrial NADH:ubiquinone oxidoreductase and induce ornithine decarboxylase, a marker for cancer chemopreventive potency, with IC<sub>50</sub> = 30  $\mu$ M (Fang & Casida, 1998). The simultaneous effects of these phytochemicals on NADH:ubiquinone oxidoreductase and F0F1-ATPase/ATP synthase could significantly affect mitochondrial function and alter ATP level, mitochondrial transmembrane potential and generation of reactive oxygen species, which have been implicated in many cellular processes such as cellular protection, apoptosis, O<sub>2</sub> sensing and ageing (Wallace, 1999). Further examinations of the effects of the phytochemicals on mitochondrial functions will, therefore, be necessary to identify precisely the mechanisms involved.

We should like to thank Dr K.E. Kwast of the Department of Molecular and Integrative Physiology and Dr W.G. Helferich of the Department of Food Science and Human Nutrition at the University of Illinois at Urbana-Champaign for critical reading and comments, Dr G. Dent of University of Southampton School of Medicine, U.K. for his advice and help in statistical analysis of data, Dr J. Clemens of Eli Lilly & Co., Indianapolis IN, for efrapentin, and Dr D. Bagchi of InterHealth Nutraceuticals, Concord, CA, for GSPE. This work was supported by an NIH grant to V.D. Ramirez.

## References

- ABRAHAMAS, J.P., BUCHANAN, S.K., VAN RAAJ, M.J., FEARNLEY, I.M., LESLIE, A.G.W. & WALKER, J.E. (1996). The structure of bovine F1-ATPase complexed with the peptide antibiotic efrapentin. *Proc. Natl. Acad. Sci. U.S.A.*, **93**, 9420–9424.
- ADLERCREUTZ, H. (1995). Phytoestrogens: epidemiology and a possible role in cancer protection. *Environ. Health Perspect.*, **103**, 103–112.
- AKIYAMA, T., ISHIDA, J., NAKAGAWA, S., OGAWARA, H., WATANABE, S.-I., ITOH, N., SHIBUYA, M. & FUKAMI, Y. (1987). Genistein, a specific inhibitor of tyrosine-specific protein kinases. *J. Biol. Chem.*, **262**, 5592–5595.
- ANTIPENKO, A.Y., SPIELMAN, A.I. & KIRCHBERGER, M.A. (1999). Interactions of 6-gingerol and ellagic acid with the cardiac sarcoplasmic reticulum Ca<sup>2+</sup>-ATPase. *J. Pharmacol. Exp. Ther.*, **290**, 227–234.
- BARNES, S. & PETERSON, T.G. (1995). Biochemical targets of the isoflavone genistein in tumor cell lines. *Proc. Soc. Exp. Biol. Med.*, **208**, 103–108.
- BOYER, P.D. (1997). The ATP synthase—a splendid molecular machine. *Ann. Rev. Biochem.*, **66**, 717–749.
- BRADFORD, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248–254.
- BRAVO, L. (1998). Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. *Nutrition Rev.*, **56**, 317–333.
- CHUN, Y.J., KIM, M.Y. & GUENGERICH, F.P. (1999). Resveratrol is a selective human cytochrome P450 1A1 inhibitor. *Biochem. Biophys. Res. Commun.*, **262**, 20–24.
- CIOLINO, H.P. & YEH, G.C. (1999). Inhibition of aryl hydrocarbon-induced cytochrome P-450 1A1 enzyme activity and CYP1A1 expression by resveratrol. *Mol. Pharmacol.*, **56**, 760–767.
- CROSS, R.L. & KOHLBRENNER, W.E. (1978). The mode of inhibition of oxidative phosphorylation by efrapentin (A23871). *J. Biol. Chem.*, **253**, 4865–4873.
- DAS, B., MONDRAGON, M.O.H., SADEGHIAN, M., HATCHER, V.B. & NORIN, A.J. (1994). A novel ligand in lymphocyte-mediated cytotoxicity: expression of the  $\beta$  subunit of H<sup>+</sup> transporting ATP synthase on the surface of tumour cell lines. *J. Exp. Med.*, **180**, 273–281.
- DASHWOOD, R.H. (1998). Indole-3-carbinol: anticarcinogen or tumor promoter in brassica vegetables? *Chem. Biol. Interact.*, **110**, 1–5.
- DEMROW, H.S., SLANE, P.R. & FOLTS, J.D. (1995). Administration of wine and grape juice inhibits in vivo platelet activity and thrombosis in stenosed canine coronary arteries. *Circulation*, **91**, 1182–1188.
- DEWICK, P.M. (1997). *Medicinal Natural Products: a Biosynthetic Approach*. John Wiley & Sons, Chichester.
- DI PIETRO, A., GODINOT, C., BOUILLANT, M.-L. & GAUTHERON, D.C. (1975). Pig heart mitochondrial ATPase: properties of purified and membrane-bound enzyme. *Biochimie*, **57**, 959–967.
- EVANS, B.A., GRIFFITHS, K. & MORTON, M.S. (1995). Inhibition of 5  $\alpha$ -reductase in genital skin fibroblasts and prostate tissue by dietary ligands and isoflavonoids. *J. Endocrinol.*, **147**, 295–302.
- FANG, N. & CASIDA, J.E. (1998). Anticancer action of cube insecticide: correlation for rotenoid constituents between inhibition of NADH:ubiquinone oxidoreductase and induced ornithine decarboxylase activities. *Proc. Natl. Acad. Sci. U.S.A.*, **95**, 3380–3384.
- FERRELL, J.E., SING, P.D.G.C., LOEW, G., KING, R., MANSOUR, J.M. & MANSOUR, T.E. (1979). Structure/activity studies of flavonoids as inhibitors of cyclic AMP phosphodiesterase and relationship to quantum chemical indices. *Mol. Pharmacol.*, **16**, 556–568.
- FEWTRELL, C.M.S. & GOMPERS, B.D. (1977). Effect of flavone inhibitors of transport ATPases on histamine secretion from rat mast cells. *Nature*, **265**, 635–636.
- FOTSIS, T., PEPPER, M., ADLERCREUTZ, H., FLEISCHMANN, G., HASE, T., MONTESANO, R. & SCHWEIGERER, L. (1993). Genistein, a dietary-derived inhibitor of in vitro angiogenesis. *Proc. Natl. Acad. Sci. U.S.A.*, **90**, 2690–2694.
- FRANKEL, E.N., WATERHOUSE, A.L. & KINSELLA, J.E. (1993). Inhibition of human LDL oxidation by resveratrol. *Lancet*, **341**, 1103–1104.
- FURHMAN, B., LAVY, A. & AVIRAM, M. (1995). Consumption of red wine with meals reduces the susceptibility of human plasma and low-density lipoprotein to lipid peroxidation. *Am. J. Clin. Nutr.*, **61**, 549–554.
- GEHM, B.D., MCANDREWS, J.M., CHIEN, P.-Y. & JAMESON, J.L. (1997). Resveratrol, a polyphenolic compound found in grapes and wine, is an agonist for the estrogen receptor. *Proc. Natl. Acad. Sci. U.S.A.*, **94**, 14138–14143.



- GOLDBERG, D.M., YAN, J., NG, E., DIAMANDIS, E.P., KARUMAN-CHIRI, A., SOLEAS, G. & WATERHOUSE, A.L. (1995). A global survey of *trans*-resveratrol concentrations in commercial wines. *Am. J. Enol. Vitic.*, **46**, 159–165.
- HARRIS, D.A. (1987). Spectrophotometric assays. In *Spectrophotometry and Spectrofluorimetry: a Practical Approach*. ed. Harris, D.A. & Bashford, C.L. pp. 49–90. Oxford: IRL Press.
- HIRANO, T., OKA, K. & AKIBA, M. (1989). Effects of synthetic and naturally occurring flavonoids on Na<sup>+</sup>, K<sup>+</sup>-ATPase: aspects of the structure-activity relationship and action mechanism. *Life Sci.*, **45**, 1111–1117.
- HOLLMAN, P.C., FESKENS, E.J.M. & KATAN, M.B. (1999). Tea flavonols in cardiovascular disease and cancer epidemiology. *Proc. Soc. Exp. Biol. Med.*, **220**, 198–202.
- HSIEH, C.-Y., SANTELL, R.C., HASLAM, S.Z. & HELFERICH, W.G. (1998). Estrogenic effects of genistein on the growth of estrogen receptor-positive human breast cancer (MCF-7) cells in vitro and in vivo. *Cancer Res.*, **58**, 3833–3838.
- JANG, M., CAI, L., UDEANI, G.O., SLOWING, K.V., THOMAS, C.F., BEECHER, C.W., FONG, H.H., FARNSWORTH, N.R., KINGHORD, A.D., MEHTA, R.G., MOON, R.C. & PEZZUTO, J.M. (1997). Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science*, **275**, 218–220.
- KING, R.A. & BURSILL, D.B. (1998). Plasma and urinary kinetics of the isoflavones daidzein and genistein after a single soy meal in humans. *Am. J. Clin. Nutr.*, **67**, 867–872.
- KUIPER, G.G.J.M., LEMMEN, J.G., CARLSSON, B., CORTON, J.C., SAFE, S.H., VAN DER SAAG, P.T., VAN DER BURG, B. & GUSTAFSSON, J.-A. (1998). Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. *Endocrinology*, **139**, 4252–4263.
- LANG, D.R. & RACKER, E. (1974). Effects of quercetin and F1 inhibitor on mitochondrial ATPase and energy-linked reactions in submitochondrial particles. *Biochim. Biophys. Acta*, **333**, 180–186.
- LINNETT, P.E. & BEECHEY, R.B. (1979). Inhibitors of the ATP synthetase system. *Methods Enzymol.*, **55**, 472–518.
- MATSUYAMA, S., XU, Q., VELOURS, J. & REED, J.C. (1998). The mitochondrial F0F1-ATPase proton pump is required for function of the proapoptotic protein Bax in yeast and mammalian cells. *Mol. Cell.*, **1**, 327–336.
- MCENERY, M.W., BUHLER, Jr, E.L., AEBI, U. & PEDERSEN, P.L. (1984). Proton ATPase of rat liver mitochondria: preparation and visualization of a functional complex using the novel zwitterionic detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate. *J. Biol. Chem.*, **259**, 4642–4651.
- MCENERY, M.W., HULLIHEN, J. & PEDERSEN, P.L. (1989). F0 'proton channel' of rat liver mitochondria: rapid purification of a functional complex and a study of its interaction with the unique probe diethylstilbestrol. *J. Biol. Chem.*, **264**, 12029–12036.
- MCENERY, M.W. & PEDERSEN, P.L. (1986). Diethylstilbestrol: a novel F0-directed probe of the mitochondrial proton ATPase. *J. Biol. Chem.*, **261**, 1745–1752.
- MCKENNA, E., SMITH, J.S., COLL, K.E., MAZACK, E.K., MAYER, E.J., ANTANAVAGE, J., WIEDMANN, R.T. & JOHNSON, R.G. (1996). Dissociation of phospholamban regulation of cardiac sarcoplasmic reticulum Ca<sup>2+</sup> ATPase by quercetin. *J. Biol. Chem.*, **271**, 24517–24525.
- MOSER, T.L., STACK, M.S., ASPLIN, I., ENGHILD, J.J., HOJRUP, P., EVERITT, L., HUBCHAK, S., SCHNAPER, H.W. & PIZZO, S.V. (1999). Angiotensin binds ATP synthase on the surface of human endothelial cells. *Proc. Natl. Acad. Sci. U.S.A.*, **96**, 2811–2816.
- MURKIES, A.L., WILCOX, G. & DAVIS, S.R. (1998). Clinical review 92: phytoestrogens. *J. Clin. Endocrinol. Metab.*, **83**, 297–303.
- PEDERSEN, P.L. & AMZEL, L.M. (1993). ATP synthase: structure, reaction center, mechanism, and regulation of one of nature's most unique machines. *J. Biol. Chem.*, **268**, 9937–9940.
- RAGAN, C.I., WILSON, M.T., DARLEY-USMAR, V.M. & LOWE, P.N. (1987). Subfractionation of mitochondria and isolation of the proteins of oxidative phosphorylation. In: *Mitochondria: a Practical Approach*. ed. Darley-USmar, V.M., Rickwood, D. & Wilson, M.T. pp. 79–112. Oxford: IRL Press.
- SERAFINI, M., MAIANI, G. & FERRO-LUZZI, A. (1998). Alcohol-free red wine enhances plasma antioxidant capacity in humans. *J. Nutr.*, **128**, 1003–1007.
- SETCHELL, K.D.R. (1998). Phytoestrogens: the biochemistry, physiology, and implications for human health of soy isoflavones. *Am. J. Clin. Nutr.*, **68**, 1333S–1346S.
- SHAO, Z.-M., ALPAUGH, M.L., FONTANA, J.A. & BARSKY, S.H. (1998). Genistein inhibits proliferation similarly in estrogen receptor-positive and negative human breast carcinoma cell lines characterized by P21WAF1/CIP1 induction, G2/M arrest, and apoptosis. *J. Cell. Biochem.*, **69**, 44–54.
- STEVENS, M.F., MCCALL, C.J., LELIEVELD, P., ALEXANDER, P., RICHTER, A. & DAVIES, D.E. (1994). Structural studies on bioactive compounds. 23. Synthesis of polyhydroxylated 2-phenylbenzothiazoles and a comparison of their cytotoxicities and pharmacological properties with genistein and quercetin. *J. Med. Chem.*, **37**, 1689–1695.
- THAM, D.M., GARDNER, C.D. & HASKELL, W.L. (1998). Clinical review 97: Potential health benefits of dietary phytoestrogens: a review of the clinical, epidemiological, and mechanistic evidence. *J. Clin. Endocrinol. Metab.*, **83**, 2223–2235.
- VAN RAAJ, M.J., ABRAHAMS, J.P., LESLIE, A.G.W. & WALKER, J.E. (1996). The structure of bovine F1-ATPase complexed with the antibiotic inhibitor aurovertin B. *Proc. Natl. Acad. Sci. U.S.A.*, **93**, 6913–6917.
- VUORINEN, K., YLITALO, K., PEUHKURINEN, K., RAATIKAINEN, P., ALA-RAMI, A. & HASSINEN, I.E. (1995). Mechanisms of ischemic preconditioning in rat myocardium: roles of adenosine, cellular energy state, and mitochondrial F1F0-ATPase. *Circulation*, **91**, 2810–2818.
- WALLACE, D.C. (1999). Mitochondrial diseases in man and mouse. *Science*, **283**, 1482–1488.
- WATANABE, S., YAMAGUCHI, M., SOBUE, T., TAKAHASHI, T., MIURA, T., ARAI, Y., MAZUR, W., WAHALA, K. & ADLER-CREUTZ, H. (1998). Pharmacokinetics of soybean isoflavones in plasma, urine and feces of men after ingestion of 60 g baked soybean powder (kinako). *J. Nutr.*, **128**, 1710–1715.
- WATKINS, T.R. (1997). *Wine: Nutritional and Therapeutic Benefits*. American Chemical Society, Washington, DC.
- WOLVETANG, E.J., JOHNSON, K.L., KRAUER, K., RALPH, S.J. & LINNANE, A.W. (1994). Mitochondrial respiratory chain inhibitors induce apoptosis. *FEBS Lett.*, **339**, 40–44.
- XU, X., HARRIS, K.S., WANG, H.J., MURPHY, P.A. & HENDRICH, S. (1995). Bioavailability of soybean isoflavones depends upon gut microflora in women. *Nutr.*, **125**, 2307–2315.
- YANG, C.S., KIM, S., YANG, G.-Y., LEE, M.-J., LIAO, J., CHUNG, J.Y. & HO, C.-T. (1999). Inhibition of carcinogenesis by tea: bioavailability of tea polyphenols and mechanisms of actions. *Proc. Soc. Exp. Biol. Med.*, **220**, 213–217.
- YE, X., KROHN, R.L., LIU, W., JOSHI, S.S., KUSZYNSKI, C.A., MCGINN, T.R., BAGCHI, M., PREUSS, H.G., STOHS, S.J. & BAGCHI, D. (1999). The cytotoxic effects of a novel IH 636 grape seed proanthocyanidin extract on culture human cancer cells. *Mol. Cell. Biochem.*, **196**, 99–108.
- ZHENG, J. & RAMIREZ, V.D. (1999a). Purification and identification of an estrogen binding protein from rat brain: oligomycin sensitivity-conferring protein (OSCP), a subunit of mitochondrial F0F1-ATP synthase/ATPase. *J. Steroid Biochem. Mol. Biol.*, **68**, 65–75.
- ZHENG, J. & RAMIREZ, V.D. (1999b). Rapid inhibition of rat brain mitochondrial proton F0F1-ATPase activity by estrogens: comparison with Na<sup>+</sup>, K<sup>+</sup>-ATPase of porcine cortex. *Eur. J. Pharmacol.*, **368**, 95–102.
- ZHENG, J. & RAMIREZ, V.D. (1999c). Piceatannol, a stilbene phytochemical, inhibits mitochondrial F0F1-ATPase activity by targeting the F1 complex. *Biochem. Biophys. Res. Commun.*, **261**, 499–503.
- ZINI, R., MORIN, C., BERTELLI, A., BERTELLI, A.A. & TILLEMENT, J.P. (1999). Effects of resveratrol on the rat brain respiratory chain. *Drugs Exp. Clin. Res.*, **25**, 87–97.

(Received December 9, 1999

Revised March 14, 2000

Accepted March 27, 2000)