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Rapid inhibition of rat brain mitochondrial proton F0F1-ATPase activity by estrogens: comparison with Na^+, K^+-ATPase of porcine cortex

Jianbiao Zheng, Victor D. Ramirez *

Department of Molecular and Integrative Physiology and Neuroscience Program, University of Illinois at Urbana-Champaign, 524 Burrill Hall, 407 South Goodwin Avenue, Urbana, IL 61801, USA

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Abstract

Our earlier studies have identified oligomycin sensitivity-conferring protein (OSCP), a subunit of proton F0F1-ATPase/ATP synthase in the mitochondrial inner membranes, as a new estradiol binding protein. This finding suggests that mitochondrial ATPase/ATP synthase could be a potential target for estradiol or compounds with similar structures. Here, we report that estradiol and several other compounds inhibited F0F1-ATPase activity of detergent-solubilized rat brain mitochondrial preparations in a following decreasing order: diethylstilbestrol (half-inhibition concentration, IC_{50} of 10–25 μM) > α-zearalenol, 4-hydroxyestradiol (IC_{50} of 55 μM) > 2-hydroxyestradiol (IC_{50} of 110 μM), 17β-estradiol, 17α-estradiol > β-zearalanol > estriol, testosterone, 16α-hydroxyestrone > corticosterone, progesterone, dehydroepiandrosterone, dehydroepiandrosterone 3-sulfate, cholesterol (less than 10% inhibition at 140 μM). On the other hand, Na^+, K^+-ATPase of porcine cortex showed different sensitivity to the compounds tested above. At 70 μM, the rank of inhibitory potency in decreasing order was as follows: 2-hydroxyestradiol (IC_{50} of 70 μM) > diethylstilbestrol > 4-hydroxyestradiol > progesterone > α-zearalenol, while other compounds had little effect (less than 5%). The data indicate that the ubiquitous mitochondrial F0F1-ATPase is a specific target site for estradiol and related estrogenic compounds; however, under this in vitro condition, the effect seems to require pharmacological concentrations. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Estrogen; Catecholestrogen; Mitochondrion; Oligomycin; F0F1-ATPase; Na^+, K^+-ATPase

1. Introduction

Estrogens exert direct actions on a variety of cells such as neurons, vascular smooth muscle cells and osteoblasts by mechanisms independent from nuclear estrogen receptors, so-called non-genomic mechanisms (McEwen, 1991; Farhat et al., 1996; Ramirez and Zheng, 1996; Moss et al., 1997). Some of these actions were revealed at estrogen concentrations close to physiological conditions (nM), but many required pharmacological concentrations (0.1–100 μM) of estrogens (e.g., Jiang et al., 1992; Behl et al., 1995; Yamamoto, 1995; review by Farhat et al., 1996; Sitzler et al., 1996; Weaver et al., 1997; Sawada et al., 1998). The detailed mechanisms of these actions are unclear at present time and there is a need to identify the estrogen targeting proteins responsible for these actions.

Recent studies from our laboratory have identified oligomycin sensitivity-conferring protein (OSCP), a subunit of proton F0F1-ATPase/ATP synthase (F-type ATPase) in the mammalian mitochondrial inner membranes, as a novel estradiol binding protein (Ramirez et al., 1996; Zheng and Ramirez, 1999). Although there is no report on the effect of estradiol or other naturally occurring estrogens on F0F1-ATPase/ATP synthase, a synthetic stilbene, diethylstilbestrol (DES), with structure similar to estradiol, had been shown before to inhibit the proton transport by F0 sector of the rat liver F0F1-ATPase as well as the activity of purified F0F1-ATPase at micromolar concentrations (McEnery and Pedersen, 1986; McEnery et al., 1989). Our studies also indicated that diethylstilbestrol and estradiol partially inhibited the binding of estradiol chemically coupled to [^{125}I]-labeled bovine serum albumin to recombinant bovine OSCP (Zheng and Ramirez, 1999). Therefore, it seems that both diethylstilbestrol and estradiol binds OSCP, suggesting that estradiol, like diethylstilbestrol, could also affect the F0F1-ATPase activity by its binding to OSCP. However, diethylstilbestrol also inhibited several other ATPases including P-type ATPases such as Ca^{2+}-ATPase (Martinez-Azorin et al., 1992), and V-type AT-

* Corresponding author. Tel.: +1-217-333-0315; Fax: +1-217-333-1133; E-mail: vdramire@staff.uiuc.edu

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Pase in the vesicles (Gronberg and Flatmark, 1988). The involvement of any subunits of the ATPases is not known at present time, although a non-specific action through membrane lipids had been suggested (Gronberg and Flatmark, 1988).

Several other steroids at 14 μM have been studied for their effects on Na^+, K^+-ATPase in plasma membranes (Alivisatos et al., 1981). In this study, estrone and progesterone were found to inhibit this ATPase and were mimicked by dodecanol and its glucoside. On the other hand, cholesterol and testosterone stimulated this ATPase and were mimicked by octanol and its glucoside. These effects were believed to be due to a non-specific effect on membrane lipids since all other integral proteins studied such as adenylate cyclase were also affected while peripheral and cytosolic proteins such as acetylcholinesterase and lactate dehydrogenase were not (Alivisatos et al., 1981). The effects also required prolonged incubation of these steroids with Na^+, K^+-ATPase preparation and only became obvious after 30 min incubation (Alivisatos et al., 1981). Related to these findings are the studies by Farnsworth (1990), who demonstrated that dihydrotestosterone at 1 nM bound to β-subunit of Na^+, K^+-ATPase, and enhanced the enzyme activity after pre-incubation.

For progesterone and its derivatives, it is also well-known that they specifically and rapidly inhibited Na^+, K^+-ATPase by directly binding to ouabain-sensitive site, and may contribute to endogenous digitalis-like activity (LaBella et al., 1979, 1985). Could estradiol and its metabolites play a similar role in F0F1-ATPase?

In this paper, we test a digitonin-solubilized brain mitochondrial preparation used earlier in our studies for purification and identification of OSCP (Zheng and Ramirez, 1999) as well as a 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)-solubilized preparation (McEnery et al., 1984) to study the effects of estrogens and several other steroids on the F0F1-ATPase activity with an ATP-regenerating NADH-coupled enzyme assay system. We report here for the first time the inhibitory actions of estradiol and several other estrogenic compounds on the F0F1-ATPase activity in vitro, identifying a novel mechanism for actions of estrogens, catecholestrogens and similar compounds that is different from the classical nuclear estrogen receptor mechanism. The effects of these compounds on the activity of Na^+, K^+-ATPase from porcine cortex were also tested and compared.

2. Materials and methods

2.1. Chemical reagents and materials

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 (1.0 $\mu g\ \mu l^{-1}$). Efrapeptin (efrastatin, A23871) 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 including porcine cortex Na^+, K^+-ATPase were purchased from Sigma (St. Louis, MO). Ouabain (stock solution of 5 $\mu g\ \mu l^{-1}$) and reduced glutathione were dissolved in sterile distilled water. All other steroids were prepared in 100% ethanol with a final concentration of 10 mM. The final methanol or ethanol concentrations in the reaction solution was usually 0.7% and occasionally 1.4%. The amount of alcohol had minimal effect on the responses ($< 5\%$). In every experiment, control trials with the same amount of vehicle (water or alcohol) were included.

2.2. Animals

Adult female Sprague–Dawley rats (60–120 days old, unknown estrous cycle) were maintained on a 14:10 h 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 and killed by rapid decapitation.

2.3. Subcellular fractionation

The following procedures were performed at 4°C. The brain mitochondrial fractions (mP2) and microsomal fractions (P3) of adult rats were prepared according to Zheng and Ramirez (1997). Briefly, brains of adult female rats were rapidly removed and homogenized in a Teflon glass homogenizer in 10 vol (original weight/vol) of ice-cold P2-Tris buffer (50 mM Tris–HCl/120 mM NaCl/5 mM KCl/1 mM $MgSO_4$/1 mM $CaCl_2$/10% glycerol, pH 7.4 at 4°C) plus 0.5 mM AEBSF and 0.1 mM bacitracin. Homogenates were centrifuged at $600 \times g$ for 10 min to spin down the nuclei and cell debris. The supernatant (S1) was centrifuged again at $15{,}000 \times g$ for 5 min to spin down the mitochondria (mP2 fraction). The supernatant obtained (S2) was centrifuged again at $125{,}000 \times g$ for 60 min to obtain microsomal fraction (P3). The pellets (mP2, P3) were resuspended in the above buffer, and stored at −80°C. The fractions were assayed for protein by the method of Bradford (1976) using bovine serum albumin as a reference.

2.4. Solubilization

Frozen mP2 or P3 fractions were thawed and solubilized in P2-Tris buffer containing 1% digitonin (Zheng and Ramirez, 1997, 1999) or 1% CHAPS (McEnery et al., 1984) at 4°C as reported.

2.5. Assay for ATPase activity

ATPase activity of solubilized brain mP2 fractions was measured spectrophotometrically at 340 nm with a Hitachi UV/Vis Spectrophotometer model U-2001 by coupling the production of ADP to the oxidation of NADH via the pyruvate kinase and lactate dehydrogenase reaction (McEnery and Pedersen, 1986; Harris, 1987). The reaction mixture contained in a final volume of 0.7 ml: 100 mM Tris (pH 8.0), 4.0 mM MgATP, 2 mM $MgCl_2$, 50 mM KCl, 0.2 mM EDTA, 0.4 mM NADH, 1 mM phosphoenol pyruvate, 1.4 unit of pyruvate kinase, 1.4 unit of lactate dehydrogenase. This assay condition minimizes the contribution of other transport ATPases such as Na^+, K^+-ATPase or Ca^{2+}-ATPase. Various testing compounds were then added 0.5 min before the reaction was started by adding 14.2–20 μl of solubilized brain membrane fractions (solubilized mP2 or P3, about 50 μg proteins) at constant

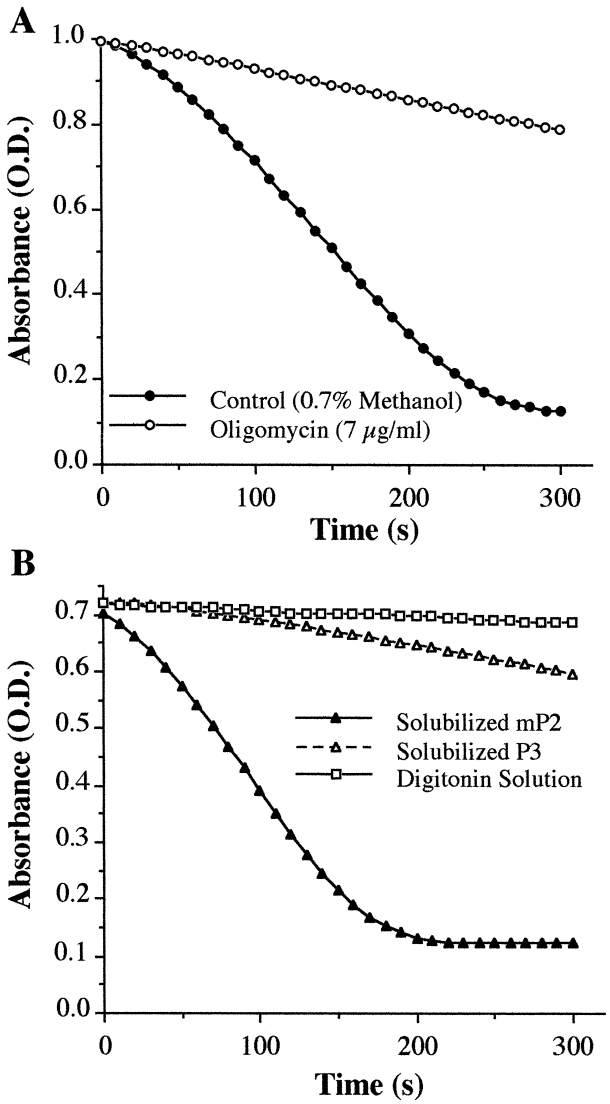

Fig. 1. Examples of spectrophotometric read-out of ATPase activity in digitonin-solubilized brain subcellular fractions. The reactions were started by the addition of 20 μl solubilized sample followed by mixing and 30 s later the data were collected every 10 s for 5 min. (A) A typical example showing the effect of oligomycin on the responses of solubilized mP2 (51 μg proteins). The control response for solubilized mP2 in 0.7% methanol was 0.246 O.D. min^{-1} and in the presence of oligomycin it was 0.044 O.D. min^{-1}. The oligomycin-sensitive ATPase activity (F0F1-ATPase activity) was determined to be 0.446 μmol ATP min^{-1} mg^{-1} protein. (B) Changes in O.D. induced by solubilized B-mP2 (51 μg protein), P3 (52 μg protein), and digitonin solution alone. The responses for solubilized mP2 and P3 were 0.230 and 0.0236 O.D. min^{-1}, respectively.

Table 1
Effects of several inhibitors on absorbance change per minute of digitonin-solubilized and CHAPS-solubilized rat brain mP2 fractions in an NADH-linked ATP regeneration system

Treatment	Concentration	Digitonin-solubilized (%)	CHAPS-solubilized (%)
Control	–	100	100
Without ATP	–	2.6 ± 0.4 (3)	ND
Boiled preparation	–	1.6 ± 1.2 (3)	ND
Oligomycin	7 μg ml^{-1}	16.5 ± 2.2 (7)	17.6
Efrapeptin	1–2 μM	16.8	17.1
Oligomycin + Efrapeptin	7 μg ml^{-1} 1 μM	17.7	ND
Oligomycin + Antimycin A	7 μg ml^{-1} 2 μg ml^{-1}	16.1	ND
Ouabain	98 μM	99.0	99.0

The control experiments were done in the presence of same amount of vehicle (methanol, ethanol or distilled water) and were expressed as 100% and the variation between control trials was less than 3%.
For some treatments, the data were expressed as means ± S.D. with the number of experiments in parentheses.
ND, not determined.

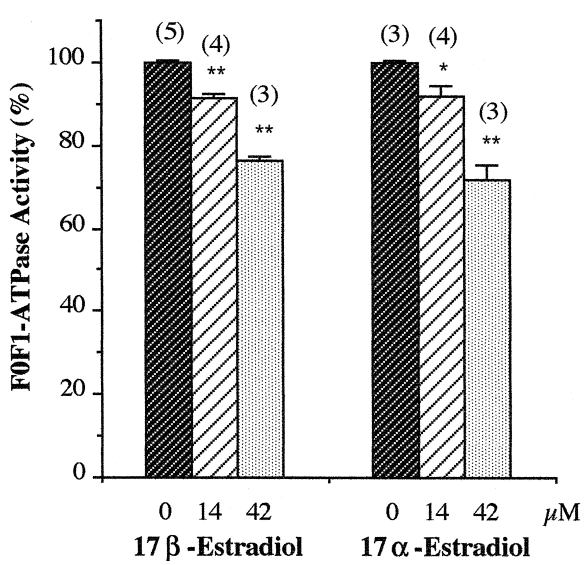

Fig. 2. Effects of 17β-estradiol and 17α-estradiol on F0F1-ATPase activity of digitonin-solubilized brain mP2. Data were expressed in means ± S.D. In parenthesis is the number of trials performed. $^{*}P < 0.01$, $^{**}P < 0.0001$ using ANOVA.

temperature of 30–31°C. For studying the effect of efrapeptin on ATPase activity, efrapeptin was pre-incubated at 26°C with solubilized preparation for 6 min to allow for binding followed by addition of both, as described earlier (Cross and Kohlbrenner, 1978). Stable values were obtained up to 6 h. The steady-state linear range of the absorbance was used to obtain the reaction rates (correlation coefficient usually of 0.998–1.000). The F0F1-ATPase activity was determined in the presence of oligomycin or efrapeptin. An extinction coefficient of 6.22 mM^{-1} cm^{-1} was used for NADH in calculating the activity in micromoles ATP hydrolyzed per minute. The digitonin-solubilized brain mP2 had F0F1-ATPase activity of about 0.3–0.55 μmol ATP hydrolyzed min^{-1} mg^{-1} protein while the CHAPS-solubilized brain mP2 had F0F1-ATPase activity of about 0.25–0.35 μmol ATP hydrolyzed min^{-1} mg^{-1} protein.

The activity of the Na^+, K^+-ATPase from porcine cortex was measured similarly except 100 mM NaCl was added. The preparation had ATPase activity of about 0.25 μmol ATP hydrolyzed min^{-1} mg^{-1} protein at 30–31°C, a value which is comparable to the one as determined by the manufacturer at 37°C (0.4 μmol ATP hydrolyzed min^{-1} mg^{-1} protein). The ouabain-insensitive ATPase activity in this preparation is less than 3% as determined by the manufacturer at 1 mM ouabain.

To study the possible effects of estrogens on coupled enzymes used in the ATPase assay, i.e., pyruvate kinase

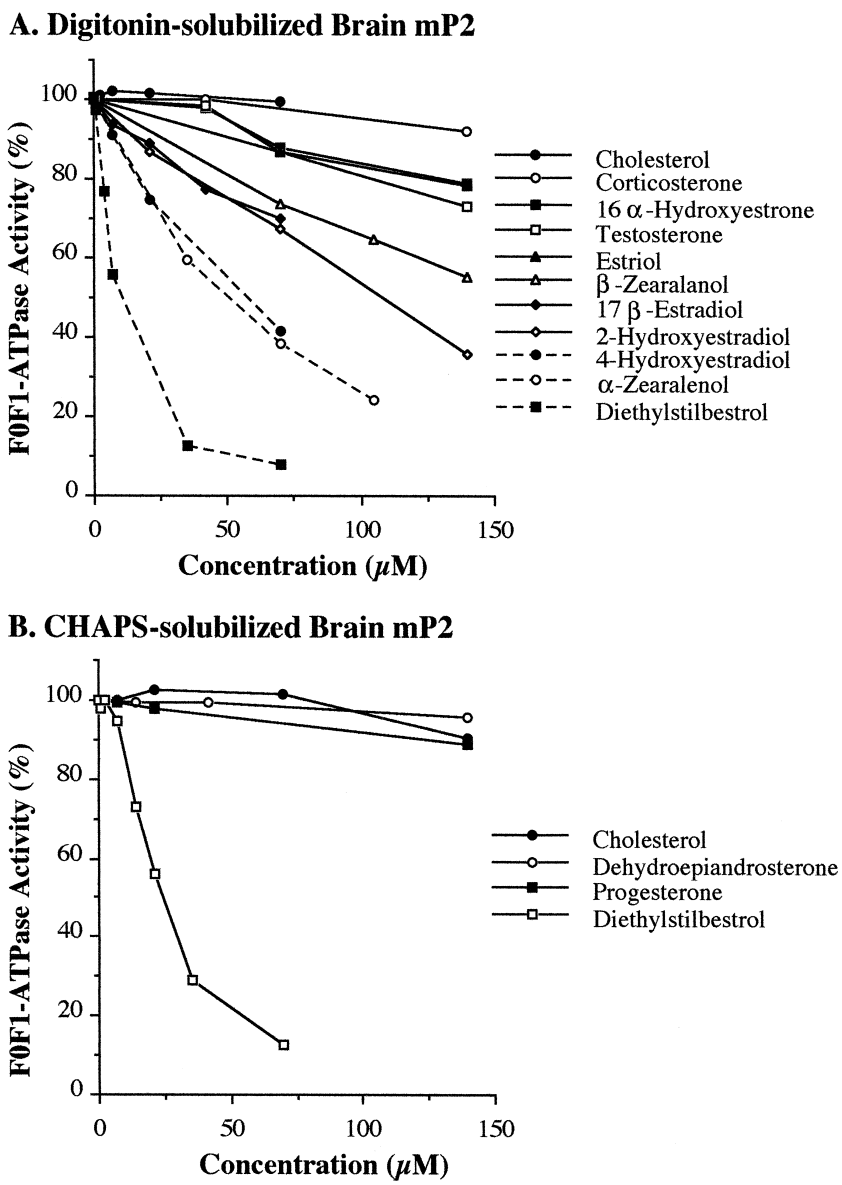

Fig. 3. Effects of several estrogens and steroids on F0F1-ATPase activity of detergent-solubilized brain mP2. (A) Digitonin-solubilized brain mitochondrial fraction. (B) CHAPS-solubilized brain mitochondrial fraction.

and lactate dehydrogenase, ATP was omitted from the buffer, and the reaction was started by adding 0.2 mM ADP (without ATPase preparation).

Since the responses were quite stable (less than 3% variation) as judged from control trials done before, during and after the experiment, single trial was used for each dosage for most experiments. Some of the data with trial numbers of three or more were also analyzed by the analysis of variance (ANOVA) between control and experimental groups and $P < 0.05$ was considered significant. In these cases, the data were expressed as means ± S.D.

3. Results

To investigate if natural estrogens might regulate the mitochondrial F0F1-ATP synthase/ATPase activity directly, we assayed the effect of free estrogens and several other steroids on the ATPase activity of solubilized brain mP2 using a coupled method as detailed in Section 2. The responses of digitonin-solubilized brain mP2 could be inhibited about 84% by 7 μg ml^{-1} oligomycin and 1–2 μM efrapeptin, or both, potent mitochondrial F0F1-ATP synthase/ATPase inhibitors (Fig. 1A and Table 1). A similar prepared solubilized B-P3 fraction had only 10% of the responses observed in solubilized B-mP2 fractions and 1% digitonin had little effect (Fig. 1B). As expected, ouabain, a specific Na^+, K^+-ATPase inhibitor, was without effect at 98 μM, although at this concentration, it inhibited the porcine cortex Na^+, K^+-ATPase by more than 95% (see below, Table 3). The NADH oxidase activity was less than 3% of the total activity as determined in the absence of ATP. Addition of antimycin A, an inhibitor of mitochondrial complex III at 2 μg ml^{-1} in the presence of oligomycin, did not further increase the inhibition (Table 1). These data indicate that most of the activity (about 85%) in the mitochondrial fraction came from efrapeptin and also oligomycin-sensitive F0F1-ATP synthase/ATPase, and was considered as mitochondrial proton F0F1-ATPase activity. The contaminated activity (about 15%) was neither efrapeptin-sensitive nor oligomycin-sensitive.

At both 14 μM and 42 μM, both 17β-estradiol and 17α-estradiol significantly inhibited F0F1-ATPase activity of solubilized brain mitochondrial fractions by 7% and 25%, respectively (Fig. 2). Application of higher concentrations of estradiol at 70 μM resulted in a clear visual precipitation of the steroid, so no further analysis was performed, though a higher inhibition was consistently observed (see Fig. 3). Several other endogenous steroids were also tested and 4-hydroxyestradiol (IC_{50} of about 55 μM) was found to be most potent (Fig. 3). The relative potency of the steroids showed a rank order of 4-hydroxyestradiol > 2-hydroxyestradiol (IC_{50} of about 110 μM), 17β-estradiol, 17α-estradiol > estriol, testosterone, 16α-hydroxyestrone > progesterone, dehydroepiandrosterone, cholesterol (little effect) (Figs. 2 and 3A and B). Dehydroepiandrosterone 3-sulfate did not affect the ATPase activity at 280 μM (not shown). Therefore, F0F1-ATPase activity was preferentially inhibited by estradiol and brain estrogen metabolites. Initial studies were also performed for progesterone and dehydroepiandrosterone in digitonin-solubilized brain mitochondrial preparation, and an increase in absorbance at 340 nm over time was consistently observed. This phenomenon, however, turns out to be due to the direct interaction of digitonin and these two steroids at micromolar concentrations, since without NADH, solubilized mitochondrial sample, ATP, and coupled enzymes, these steroids still interact with digitonin to induce the increase in absorbance. No further study was performed to investigate the possible mechanism involved in this phenomenon. The study of actions of these two steroids on F0F1-ATPase was, therefore, done in CHAPS-solubilized preparation. Two mycoestrogens also inhibited the F0F1-ATPase with α-zearalenol (IC_{50} of 50 μM) about three- to four-fold more potent than β-zearalanol (Fig. 3A). The artificial stilbene estrogen, diethylstilbestrol, which is an agonist with very high affinity for nuclear estrogen receptors (K_d of about 0.1 nM) and also shown before as an inhibitor for many types of ATPase including rat liver F0F1-ATPase at micromolar concentrations, was the most potent inhibitor among the compounds currently tested with IC_{50} of about 10 μM for digitonin-solubilized brain mitochondrial fractions (Fig. 3A) and 25 μM for CHAPS-solubilized brain mitochondrial fractions (Fig. 3B). Effects of these estrogens are on the F0F1-ATPase, and not on the oligomycin or efrapeptin-insensitive portion of the total activity, since they did not further inhibit the responses in the presence of oligomycin (not shown).

The effects of these estrogens were not due to actions on the coupled system used to measure the ATPase activity, since at similar concentrations they had no or little effect on ADP-induced responses (Table 2).

Table 2
Effects of several estrogens on ADP-induced response in the coupled assay system

Treatment	Concentration	Absorbance change (%)
Control	–	100
Diethylstilbestrol	21 μM	98.9 ± 2.0 (3)
Estradiol	42 μM	101.5 ± 0.6 (3)
Estriol	70 μM	101.8
4-Hydroxyestradiol	70 μM	100.0
2-Hydroxyestradiol	70 μM	97.8
α-Zearalenol	70 μM	95.6
β-Zearalanol	70 μM	100.0

The control experiments were done before, during, and at the end of trials to ensure consistent responses.
In some cases when three or more trials were performed, the data were expressed as means ± S.D. with the number of experiments in parentheses.

Table 3
Effects of several ATPase inhibitors and selected compounds on Na^+, K^+-ATPase of porcine cortex

Treatment	Concentration	ATPase activity (%)
Control	–	100
Without NaCl	–	1.1
Ouabain	98 μM	4.3
Oligomycin	7 μg ml^{-1}	12.8
Digitonin	0.2 mg ml^{-1}	19.4
Diethylstilbestrol	70 μM	55.9
17β-Estradiol	70 μM	103.4
17α-Estradiol	70 μM	96.4
Estriol	70 μM	103.6
2-Hydroxyestradiol	70 μM	50.9 ± 2.0 (3)
4-Hydroxyestradiol	70 μM	73.4
β-Zearalanol	70 μM	97.3
α-Zearalenol	70 μM	84.7
Cholesterol	70 μM	99.1
Dehydroepiandrosterone	70 μM	99.5
Corticosterone	70 μM	94.6
Testosterone	70 μM	95.5
Progesterone	70 μM	79.2 ± 1.1 (3)

In some cases when three or more trials were performed, the data are expressed as means ± S.D. with the number of experiments in parentheses.

Since progesterone and its derivatives showed digitalis-like activity (LaBella et al., 1979, 1985), we decided to test if estradiol and other estrogens could also affect the Na^+, K^+-ATPase. As shown in Table 3, when tested at 70 μM, 2-hydroxyestradiol (IC_{50} of 70 μM) was the most potent inhibitor of the Na^+, K^+-ATPase of porcine cortex among all the compounds used. Diethylstilbestrol, 4-hydroxyestradiol and α-zearalenol also had an inhibitory action, though less potent than 2-hydroxyestradiol. Consistent with the findings by other researchers, progesterone also inhibited this ATPase. Other compounds including testosterone, corticosterone, dehydroepiandrosterone, cholesterol, β-zearalanol, 17α-estradiol, 17β-estradiol, and estriol had little or no effect. Therefore, the sensitivity of F0F1-ATPase to estrogens and related compounds are different from Na^+, K^+-ATPase. While F0F1-ATPase is preferentially targeted by estrogens, the Na^+, K^+-ATPase could be regulated by both progesterone derivatives and estrogens, though with different sensitivity.

4. Discussion

By using a functional ATPase assay, we showed that estradiol derivatives or estrogenic compounds preferentially inhibited the rat brain mitochondrial F0F1 proton ATPase, the ubiquitous energy master of the cells. The observed inhibitory effect of estrogens on the ATP hydrolysis of solubilized mitochondrial preparations was due to the F-type ATP synthase/ATPase, and not to other contaminants. First, the mP2 preparation consisted of mainly mitochondria with little contamination by other organelles as judged by electron microscopic observations (Zheng and Ramirez, 1997). Therefore, the ATPase activity contamination from microsomal fraction (P3) is minimal, since even the solubilized P3 had ATPase activity of only 10% or less of the solubilized mP2 preparation. Second, the contribution of NADH oxidase to the measured activity was also negligible, since without ATP, only about 3% of the activity was observed. Third, the residual ATP hydrolysis activity in the presence of oligomycin/efrapeptin (about 15%) was not affected by the steroids used. Fourth, 17β-estradiol and other compounds with inhibitory action on ATPase activity had no effect on ADP-induced response (95.6–101.8% of control). Therefore, the inhibitory effect of estrogens on ATPase activity of solubilized mP2 was not due to its action on lactate dehydrogenase or pyruvate kinase used in the coupled assay. This same assay was also used in the studies of interaction between diethylstilbestrol and F0 sector by Pedersen's group (McEnery and Pedersen, 1986; McEnery et al., 1989). Fifth and most importantly, up to 85% of activity could be inhibited by oligomycin, efrapeptin, or both, F0F1-ATPase inhibitors (Linnett and Beechey, 1979); hence, the main activity could not be due to the other two types of ATP-driven cation pumps, P-type ATPases, which are efrapeptin-insensitive (Susa and Lardy, 1975), and V-type ATPases, which are oligomycin-insensitive (Forgac, 1989). Furthermore, the activity was not inhibited by ouabain, a specific Na^+, K^+-ATPase. Although oligomycin B could inhibit Na^+, K^+-ATPase from rat brain (Susa and Lardy, 1975) and oligomycin (mixture of A, B, C) used in this report also inhibited Na^+, K^+-ATPase from porcine cortex (Table 3), the Na^+, K^+-ATPase of rat brain was not sensitive to efrapeptin (Susa and Lardy, 1975), indicating that the contaminated Na^+, K^+-ATPase was essentially very little. In addition, the assay condition was not favorable for Na^+, K^+-ATPase activity or Ca^{2+}-ATPase activity since little sodium or Ca^{2+} was in the initial assay buffer (small amount Na^+ will come from solubilized mitochondrial preparation, i.e., 3.4 mM) and 0.2 mM EDTA was also present to chelate Ca^{2+}. In the case of digitonin-solubilized mitochondria, digitonin will also inhibit the Na^+, K^+-ATPase, with 80% inhibition at 0.02% digitonin (Table 3). All these data indicate that the mitochondrial ATP synthase/ATPase was responsible for the observed effects.

Since the detergent-solubilized preparations were used and the effects by estrogens were observed with less than 1–2 min after the mixing of ATPase preparation and chemical reagents in the buffer during the recording, the possibility that these rapid actions are mediated by non-specific effects on membrane lipid structure could be excluded. Since OSCP subunit of F0F1-ATPase has been identified as a weak estradiol binding protein (Zheng and Ramirez, 1999), it is most likely that the effect of estrogens is mediated by their binding to OSCP, though the involvement of sites different from OSCP in F0F1-ATPase could not be excluded.

The effect of DES on rat brain F0F1-ATPase is consistent with early studies by Pedersen' group on F0 sector of rat liver mitochondrial ATPase as well as purified F0F1-ATPase reconstituted in liposomes (McEnery and Pedersen, 1986; McEnery et al., 1989). Among other compounds tested, catecholestrogens (4-hydroxyestradiol and 2-hydroxyestradiol) and mycoestrogen (α-zearalenol and β-zearalanol) were most potent on mitochondrial F0F1-ATPase activity. Both 17β-estradiol and 17α-estradiol inhibited the F0F1-ATPase activity in the low micromolar range with similar activity. This is, therefore, different from our early purification study in that 17β-estradiol–BSA columns retained much higher amount (about 20 times) of OSCP and other subunits of ATPase than 17α-estradiol–BSA columns (Zheng and Ramirez, 1999). The reason is not clear yet but it is likely that binding affinity may not parallel the enzymatic inhibitory action. The effect of 17α-estradiol is particularly interesting, since like 17β-estradiol, it had been shown to induce an endothelium-independent and nuclear estrogen receptor-independent relaxation of pig coronary arteries with IC_{50} of 2–15 μM (Salas et al., 1994). Both 17α-estradiol and 17β-estradiol at pharmacological concentrations (10 μM) also exerted neuroprotective effects on murine hippocampal HT-22 cells by a nuclear estrogen receptor-independent mechanism (Behl et al., 1995, 1997). However, the neuroprotection effects of both estradiol isomers on SK-N-SH cells were significant near physiological concentrations (0.2 and 2 nM) (Green et al., 1997). This discrepancy for the concentrations required has been explained by a recent report that the low concentration of these two estratrienes requires the presence of reduced glutathione in the medium of the cell culture (Green et al., 1998), since in the absence of glutathione, the IC_{50} increase from about 5 nM to micromolar range in agreement with the work of Behl et al. (1995, 1997). We also studied the effect of 286 nM 17α-estradiol on the brain mitochondrial F0F1-ATPase activity in the presence of reduced glutathione (5 μM), but did not observe any effect ($99.3 \pm 2.5\%$ compared with control $100 \pm 2.2\%$ in means ± S.D., each group with three trials). Glutathione at 5–1000 μM alone did not affect the ATPase activity ($< 3\%$) (not shown). Since in the current assays, we only tested the rapid effect, it is possible that prolonged incubation with estradiol might change the effective concentrations of the steroids, especially for in vivo condition.

The inhibitory effect of estrogens on mitochondrial proton ATPase is different from the Na^+, K^+-ATPase in that the Na^+, K^+-ATPase was preferentially inhibited by progesterone derivatives at micromolar concentrations (LaBella et al., 1985). However, we showed here that Na^+, K^+-ATPase was also inhibited by 2- or 4-hydroxyestradiol and α-zearalenol, as well as diethylstilbestrol. It is not known if this effect of these estrogens is mediated by ouabain-binding sites on Na^+, K^+-ATPase as for progesterone and its derivatives.

How will the inhibition of mitochondrial F0F1-ATPase by pharmacological concentrations of estrogens affect the cell function? This inhibitory property of estrogens on the mitochondrial F0F1-ATPase is quite similar to the endogenous F1 inhibitor protein (IF1), a small peptide with molecular weight of about 10 kDa that binds the F1 sector of F0F1-ATPase and inhibits the ATPase activity without effect on ATP synthase activity of this enzyme (Rouslin, 1991). The IF1 has been implicated in protecting the cells such as cardiac muscle cells from injury during ischemia by inhibiting the F0F1-ATPase activity, therefore preserving ATP (Rouslin, 1991). It is conceivable that the inhibitory actions of estrogens on F0F1-ATPase could have similar function in protecting cell injury. It is not known if estrogens also affect the ATP synthase activity of this enzyme, however, oligomycin, which inhibits both ATPase and ATP synthase activity of the enzyme, has also been shown to be protective during ischemia (Vuorinen et al., 1995). Aside from cell protection, oligomycin at higher concentrations has also been shown to be cytotoxic for the cancer cell proliferation and has been shown to inhibit tumor growth (Dorward and Singh, 1996). Therefore, a combinatory effect of estrogens on F-type ATPase, leading to protective actions and antitumor property, could be a facet out of many beneficial effects of estrogens.

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