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Effect of Estradiol, Diethylstilbestrol, and Resveratrol on F0F1-ATPase Activity from Mitochondrial Preparations of Rat Heart, Liver, and Brain

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The question of whether estrogens or estrogen-like compounds would alter differentially the enzymatic activity of the F0F1-ATPase was addressed. Mitochondrial fractions of the liver, brain, and heart were obtained from adult male rats and solubilized by digitonin. About 85% of the adenosine triphosphate hydrolysis by these three preparations come from the mitochondrial F0F1-ATPase. The enzymatic activity differed in the following order: liver < brain < heart. A concentration of 13 n*M* estradiol stimulated the F0F1-ATPase activity in heart by 10% ($p < 0.01$), but not in liver or brain. 17β-estradiol competed off the binding of estradiol-17β-17-(*O*-carboxymethyl)oxime:^{125}I-labeled bovine serium albumin to mitochondrial preparations of the heart, revealing two binding sites. Resveratrol inhibited the F0F1-ATPase activity in both heart and liver with an IC_{50} of 13–15 μ*M*, which confirmed our previous report in preparations of brain. Lower doses (picomolar to nanomolar) of resveratrol stimulated the F0F1-ATPase activity in liver by 10% but not in heart. At 6.7 μ*M*, diethylstilbestrol (DES) inhibited the F0F1-ATPase activity in the three preparations by 61–67%. This study demonstrates that estradiol activates rat heart mitochondrial F0F1-ATPase at physiologic concentrations and that the F0F1-ATPase activity is markedly different in rat liver, brain, and heart. In addition, estradiol, DES, and resveratrol alter the F0F1-ATPase activity selectively, probably via different mechanisms.

Key Words: Estradiol; diethylstilbestrol; resveratrol; F0F1-ATPase/adenosine triphosphate synthase; mitochondria.

Introduction

Steroids primarily affect the transcription of mRNA and thus protein synthesis after interacting with intracellular nuclear receptors *(1–3)*. These genomic steroidal effects may become evident between 7.5 and 30 min after the application of steroid *(4,5)*. However, in some cases, steroidal effects occur as quickly as between a few seconds and 1 to 2 min *(6,7)*. In addition, inhibitors of DNA transcription or protein synthesis cannot block these effects *(8,9)*. These two facts are two important criteria to classify these steroidal effects as nongenomic *(10)*.

The nongenomic action of 17β-estradiol has been demonstrated in several cells and tissues, such as chondrocytes, osteoblastic cells, hippocampus, pulmonary vascular smooth muscle cells, liver, and uterus *(9,11–14)*. The nongenomic action may be mediated by hormone binding to membrane receptors, enzymes, or ion channels *(5)*. During recent years, evidence has been provided suggesting a nongenomic action of estradiol involving a putative membrane receptor and activation of second messengers such as G protein(s) coupled to cyclic adenosine monophosphate–dependent phosphorylation events *(15–17)* or to other transignal pathways *(12)*.

Previous studies from our laboratory and others have demonstrated the existence of specific binding sites for progesterone, estrogen, and testosterone in neural membranes *(18–20)*. Further, Moats and Ramirez *(21,22)* demonstrated a membrane-mediated specific uptake and translocation of estradiol-17β-6-(*O*-carboxymethyl)oxime:^{125}I-labeled bovine serum albumin (BSA) (E-6-^{125}IBSA) and of estrogen-BSA:colloidal gold conjugate from the plasma membrane to the mitochondria and lysosomes in vivo in female rats and in vitro in Hep G2 cells *(21,22)*. In 1996, Ramirez and Zheng *(23)* found that using a 17β-estradiol-coupled affinity column in the purification of a mitochondrial preparation from rat brain resulted in a significant retention of oligomycin-sensitivity conferring protein (OSCP), a subunit of mitochondrial F0F1-ATPase/adenosine triphosphate (ATP) synthase.

OSCP is one of the proteins that form the stalk region between F0 and F1 subunits of F0F1-ATPase/ATP synthase in bacteria, chloroplasts, and mitochondria *(24)*. F0F1-ATPase/ATP synthase is a key enzyme regulating oxidative phosphorylation *(25,26)*. The normal function of the F1 unit is to catalyze the synthesis of ATP, and the F0 unit is the proton channel of the complex. Solubilized F1 also exhibits ATPase activity *(25)*.

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Further study from our laboratory demonstrated that estrogens inhibited ATP hydrolysis by the F0F1-ATPase in mitochondrial preparations of rat brain at micromolar concentrations, and this inhibitory effect might be mediated by the binding of estrogens to OSCP *(27)*. However, the effect of physiologic concentrations of estradiol was not tested in these preparations, nor in the mitochondrial preparations from other tissues.

Previously, McEnery and Pedersen *(28)* reported the targeting of diethylstilbestrol (DES), an artificial estrogen, to the F0F1-ATPase in rat liver. Recently, resveratrol, a phytoestrogen, was found to be able to function as an estrogen agonist in many tissues. For example, in vitro studies provided evidence that resveratrol stimulated the proliferation of estrogen-dependent breast cells *(29)*, stimulated the proliferation of and differentiation in osteoblastic cells *(30)*, and induced the secretion of prolactin in a pituitary tumor cell line *(31)*. Similar to resveratrol, many other phytoestrogens also have estrogenic and/or antiestrogenic activity *(32)* and play a role in the prevention of estrogen-related health conditions, such as cardiovascular disease and cancer *(33)*. Since resveratrol has all these estrogen-like functions, it was of interest to test whether it also affects F0F1-ATPase function at different concentrations in different tissues.

Therefore, since estrogen can bind to the OSCP subunit of F0F1-ATPase/ATP synthase with a high affinity *(23)*, this study examined the hypothesis of whether estrogens and phytoestrogen will have a differential effect on the catalysis of rat liver, brain, and heart mitochondrial F0F1-ATPase in crude mitochondrial preparations.

Results

Validation of Pyruvate Kinase/Lactate Dehydrogenase–Coupled ATPase Assay

In control experiments using mitochondrial preparations of 25 µg of fresh liver (P2 fractions), a linear decrease in the absorbance at 340 nm (A_{340}) was observed (not shown) and the change of A_{340} per minute (ΔA_{340}/min) was about 0.043/min (Fig. 1). As expected, when the liver P2 fractions were boiled or when PK and lactate dehydrogenase (LDH) were omitted from the reaction solution, almost no change in absorbance was observed (Fig. 1).

Oligomycin is known to inhibit the mitochondrial F0F1-ATPase/ATP synthase activity *(34)*. Efrapeptin is also a potent and specific mitochondrial F0F1-ATPase/ATP synthase inhibitor *(35)*. Using the PK/LDH-coupled ATPase assay, it was shown previously that 7 µg/mL of oligomycin or 1 µ*M* efrapeptin, or both, inhibited about 84% of the ATPase activity in digitonin-and 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS)-solubilized brain P2 fractions *(27)*. In the present study, 1.3 µ*M* efrapeptin reduced the ATPase activity in digitonin-solubilized heart P2 (DHP2) or liver P2 (DLP2) fractions by about 85 and 88%, respectively (Fig. 2). These results confirmed the previous observation and indicate that about 84–88% of the ATPase activity in the brain, heart, or liver mitochondrial preparations is efrapeptin sensitive.

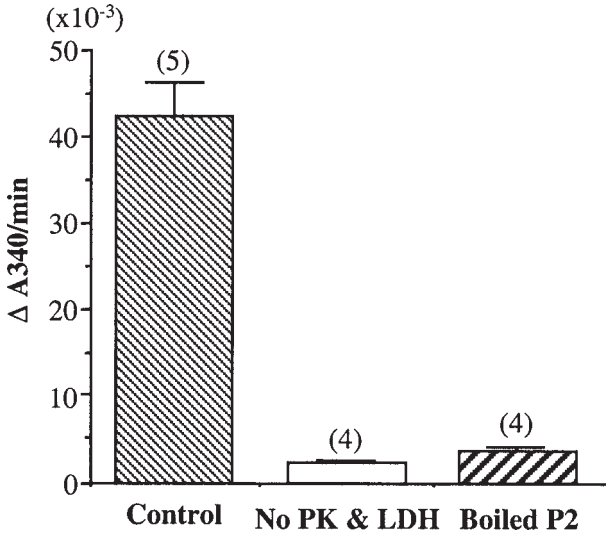

Fig. 1. Effects of different conditions on the coupled ATPase assay. Liver P2 fractions containing 25 µg of protein were tested. Control group ($n = 5$) included all the compounds for the coupled assay (see Materials and Methods). When PK and LDH were omitted from the solution or the liver P2 fractions were boiled before use, almost no ATP hydrolysis was detected ($n = 4$). Means ± SE are shown here and in subsequent figures.

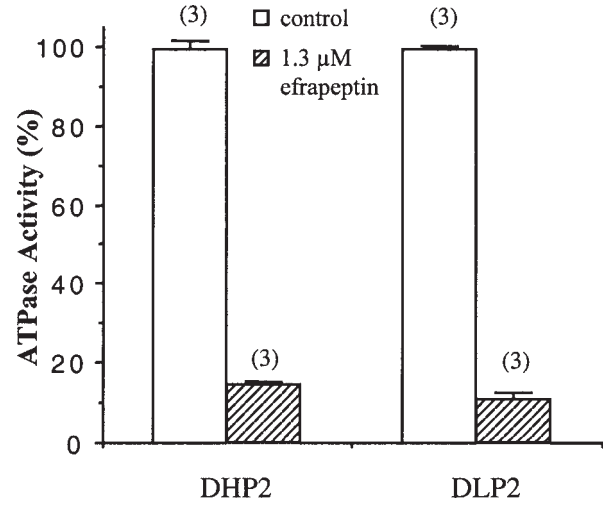

Fig. 2. Efrapeptin inhibited the ATPase activity in mitochondrial preparations of heart and liver. Efrapeptin was incubated with DHP2 or DLP2 fractions (15 µg of proteins) on ice for 6 min followed by the addition of either to start the reaction. The percentages of ATPase activity in DHP2 and DLP2 were calculated from the corresponding controls, which were considered as 100% ($n = 3$).

Figure 3 depicts the ATP concentration dependence of ATP hydrolysis. ATP caused a dose-dependent increase in ATP hydrolysis with a half-maximal increase ($K_{0.5}$) of

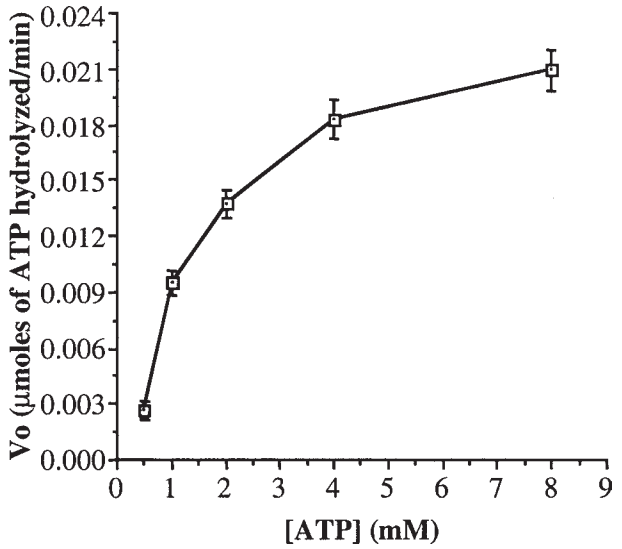

Fig. 3. Concentration dependence of ATP on ATP hydrolysis. DLP2 fractions (5 μg proteins) were used to start the ATP hydrolysis reaction. Concentrations of ATP were varied from 0.5 to 8 m*M*, and the initial velocity (micromoles of ATP hydrolyzed/min) increased accordingly ($n = 6$).

Table 1
Comparison of ATPase Activities in DLP2, DBP2, and DHP2 Fractions

Sample[a]	ATPase activity (μmol of ATP hydrolyzed/[min·mg])[b]
DLP2	0.32 ± 0.005 (15)
DBP2	1.0 ± 0.09 (15)
DHP2	2.6 ± 0.11 (24)

[a] Each sample tested contained 15 μg of protein.
[b] Numbers of experiments are shown in parentheses. Data are mean ± SE.

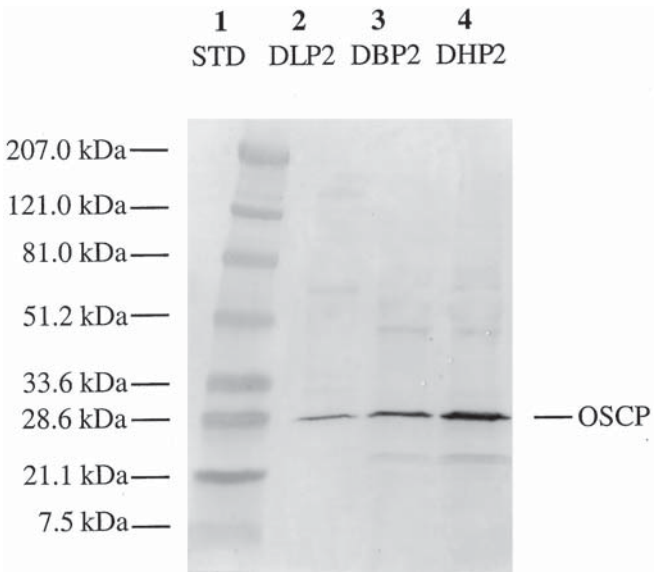

Fig. 4. Comparison of anti-OSCP antibody-binding proteins from DLP2 (lane 2), DBP2 (lane 3), and DHP2 (lane 4) using immunoblotting analysis. Each P2 preparation contained 25 μg of protein and was applied to lanes 2–4. The prestained standards (STD, lane 1, 6 μg) were myosin, 207 kDa; β-galactosidase, 121 kDa; BSA, 81 kDa; ovalbumin, 51.2 kDa; carbonic anhydrase, 33.6 kDa; soybean trypsin inhibitor, 28.6 kDa; lysozyme, 21.1 kDa; and aprotinin, 7.5 kDa. The protein around 28 kDa (corresponding to OSCP as confirmed by N-terminal protein sequencing, see text for details) had a reaction with anti-OSCP antibody in the following increasing order of intensity: DLP2 < DBP2 < DHP2.

1.5 m*M*. When DLP2 instead of intact liver P2 fractions were used, a faster reaction was observed (data not shown). This result indicated that solubilization of the P2 fractions by digitonin exposed more mitochondrial F0F1-ATPase to the reaction solution. Thus, the digitonin-solubilized P2 preparations were employed thereafter.

Interestingly, the study also revealed that the F0F1-ATPase potency of the three preparations from liver, brain, and heart was different from each other (Table 1). More specifically, DHP2 had the highest F0F1-ATPase activity (2.6 μmol of ATP hydrolyzed/[min · mg]), followed by digitonin-solubilized brain P2 fractions (DBP2) (1.0 μmol of ATP hydrolyzed/[min · mg]), while DLP2 had the lowest F0F1-ATPase activity (0.3 μmol of ATP hydrolyzed/[min · mg]). The possibility that DHP2 contains higher amounts of the ATPase was tested by Western blot of the three samples. The anti-OSCP antibody recognized OSCP protein bands in the following increasing order of intensity: DLP2 < DBP2 < DHP2 (Fig. 4). Although the molecular size of the bands recognized by the polyclonal anti-OSCP antibody seems to be ~28 kDa, different from the calculated molecular size from OSCP cDNA sequence (21 kDa) *(36)*, N-terminal protein sequencing of the first 10 amino acids of this band confirmed it to be OSCP (see Fig. 5). The difference in the observed molecular size may be owing to a change in electrophoresis migration rate caused by salt concentrations in the preparations. The relative intensities of the immunoreactive proteins analyzed by NIH IMAGE program show a ratio of 1:2:4, indicating a greater number of OSCP in the heart preparation. An electron microscopy analysis showed that the P2 pellets from the liver, brain, and heart mainly consist of lysosomes and ruptured and intact mitochondria, as determined before in brain P2 *(37)*. The electron microscopic pictures revealed that the structure of the mitochondria varied among these three P2 pellets. Heart P2 pellet (Fig. 6C) contained large mitochondria with very well-defined cristea structure compared with brain and liver P2 (Fig. 6B and 6A, respectively). The overall data indicate that DHP2 or DBP2 fractions, with eight- or threefold greater ATPase activity, are more potent preparations than DLP2.

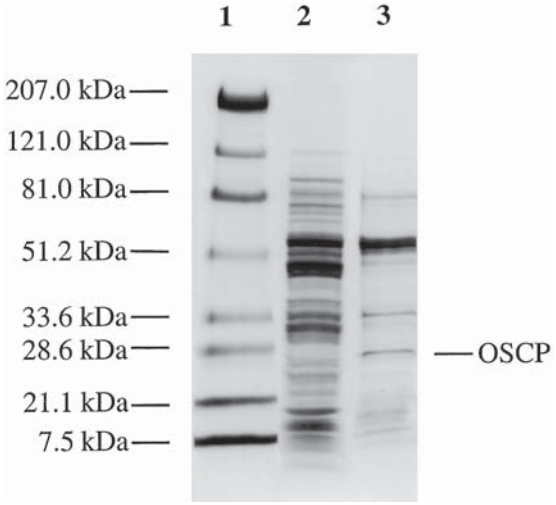

Fig. 5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of DHP2 fraction (lane 2) and estrogen-binding proteins after affinity chromatography (lane 3). Ten and 2 μg of proteins were used on lanes 2 and 3, respectively. The prestained standards (STD, lane 1, 6 μg) were myosin, 207 kDa; β-galactosidase, 121 kDa; BSA, 81 kDa; ovalbumin, 51.2 kDa; carbonic anhydrase, 33.6 kDa; soybean trypsin inhibitor, 28.6 kDa; lysozyme, 21.1 kDa; and aprotinin, 7.5 kDa. A strong band at about 28 kDa is shown in the purified DHP2 sample (lane 3). This band after N-terminal sequencing showed 100% homology with OSCP. The other proteins most likely correspond to the other subunits of the F0F1-ATPase because N-terminal sequencing of some of them from a similar brain preparation identified other subunits of the ATPase (see text for details).

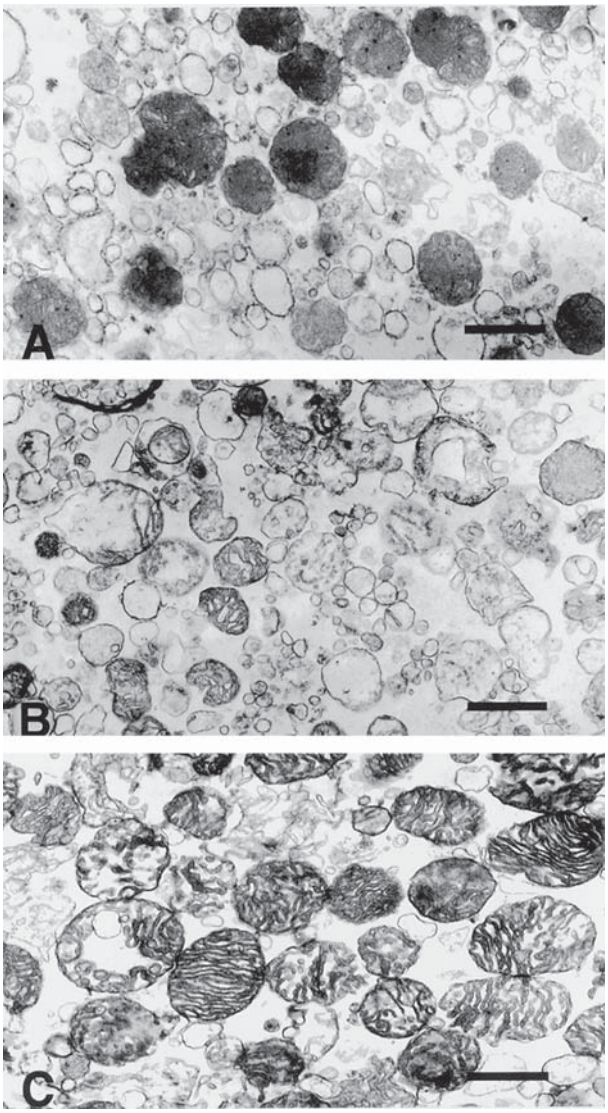

Fig. 6. Electron microscopic pictures of the P2 pellets from (**A**) liver, (**B**) brain, and (**C**) heart. The three P2 pellets mainly consist of lysosomes and mitochondria. The apparent number of mitochondria is highest in the heart P2 pellet followed by brain P2 and then liver P2. The heart P2 pellet contained mitochondria with very prominent cristea structure compared with brain and liver P2. The liver mitochondria showed a darker image, suggesting changes in the metabolic activity. Magnification: ×24,000. Bars: 0.5 μm.

Effect of Estradiol on ATP Hydrolysis by F0F1-ATPase from P2 Preparations

As shown in Fig. 7, physiologic concentrations of estradiol stimulated the ATPase activity from DHP2 compared with that of controls (with ethanol): a concentration of 1.3 n*M* increased the activity by about 6–7%, and 13 n*M* increased the activity by about 10% (student's *t*-test, $p < 0.01$ for both cases). The stimulatory effect was not observed in DBP2 or DLP2 preparations. Two-way analysis of variance (ANOVA) showed that the effect of 13 n*M* estradiol was significantly different ($p < 0.01$) among the heart, brain, and liver preparations (15 μg of proteins) but not that of 1.3 n*M*.

To test that the observed effect of 1.3 or 13 n*M* estradiol in the DHP2 preparation was owing to activation of the mitochondrial F0F1-ATPase and not to other contaminating enzymes, the effect of estradiol was examined again but in the presence of efrapeptin. Recall that efrapeptin, a specific mitochondrial F0F1-ATPase inhibitor, inhibited about 85% of the ATPase activity in DHP2 at a concentration of 1.3 μ*M*. In the presence of the same amount of efrapeptin, the addition of 1.3 or 13 n*M* estradiol did not increase the DHP2 ATPase activity (Table 2). This finding indicates that estradiol modifies the efrapeptin-sensitive F0F1-ATPase activity in the DHP2 preparation.

An inhibitory effect of estradiol at micromolar levels on the activity of F0F1-ATPase in rat brain mitochondria was observed in a previous study from our laboratory *(27)*. In the present study, 67 μ*M* estradiol inhibited the activity of F0F1-ATPase from rat heart in all three preparations, brain, or liver by about 12–20% (Table 3). Higher concentrations of estradiol could not be tested because of the solubility of the compound. Note that DES is at least 10-fold more potent than estradiol in inhibiting the ATPase activity and

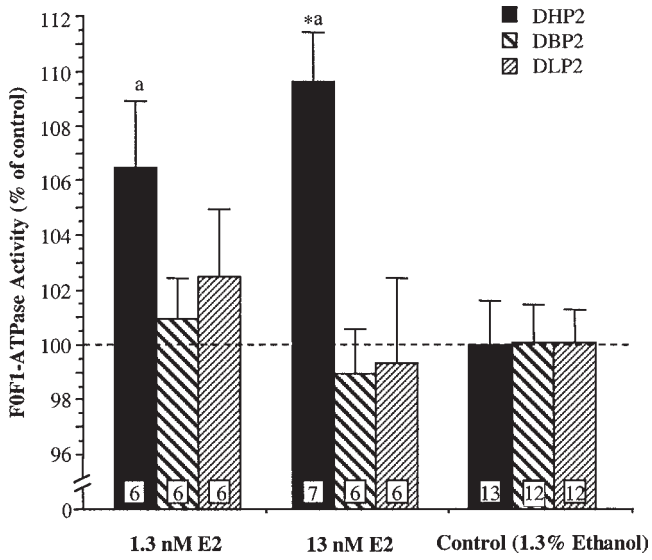

Fig. 7. Effect of estradiol (E2) on ATPase activity from mitochondrial fractions of DHP2, DBP2, or DLP2. Each fraction contained 15 μg of proteins. A concentration of 1.3 n*M* estradiol stimulated the ATPase activity from DHP2 by about 6 to 7%, but it did not have a significant effect on ATPase activity from DBP2 and DLP2. A concentration of 13 n*M* estradiol stimulated the ATPase activity from DHP2 by about 10%, while the stimulatory effect was not observed in DBP2 or DLP2 fractions. ANOVA showed that the effect of 1.3 n*M* estradiol was not significant among the three samples; however, the effect of 13 n*M* estradiol was highly significant (*$p < 0.01$). $^a p < 0.01$ vs controls. The numbers of assays corresponding to experimental (E-treated) and control (vehicle-treated) groups are indicated at the bottom of each column. The experiments were repeated three times using three different DHP2 preparations, each preparation was from a pool of tissues from two to three rats, and two to three assays were performed per preparation for each dose of estradiol. The variations between assay and between preparation were both <3% in this study.

that it lacks a stimulatory effect at 1.3 n*M* in the DHP2 preparation.

Effect of DES and Resveratrol on ATP Hydrolysis by F0F1-ATPase from P2 Preparations

The results from the present study show a potent inhibitory effect of the synthetic estrogen, DES, on the activity of F0F1-ATPase from DHP2, DBP2, and DLP2 (66, 61, and 66% inhibition with 6.7 μ*M* DES, respectively) (Table 3), confirming previous reports using a liver preparation *(28)*. In the heart preparation, 1.3 n*M* DES inhibited the F0F1-ATPase activity by about 5.8% ($p < 0.01$), which was not observed in the brain or liver preparation (Table 3).

Resveratrol belongs to the phytoestrogen family and has an estrogen-like structure similar to that of DES. The effect of resveratrol on the F0F1-ATPase was studied in DLP2 and DHP2. Figure 8 shows that resveratrol, at micromolar concentrations, inhibited the F0F1-ATPase activity in both DLP2 and DHP2 and showed an IC_{50} of about 13-15 μ*M*. A similar inhibitory effect of resveratrol on the F0F1-ATPase of DBP2 fraction was also reported recently *(38)*. Curiously, low doses of resveratrol (133 p*M* and 1.3 n*M*) stimulated the F0F1-ATPase activity in DLP2 by about 10% ($p < 0.05$) but not in DHP2 (Fig. 8).

Table 2
Effect of Estradiol (1.3 or 13 n*M*) on ATPase Activity of DHP2 Fractions (15 μg Protein) in Presence of 1.3 μ*M* Efrapeptin

Treatment	Concentration	ATPase activity (%)[b]
Control[a]	—	100 ± 1.8 (7)
Efrapeptin	1.3 μ*M*	14.9 ± 0.2 (6)[c]
Efrapeptin + Estradiol	1.3 μ*M* 1.3 n*M*	14.5 ± 0.3 (3)[c]
Efrapeptin + Estradiol	1.3 μ*M* 13 n*M*	15.0 ± 0.5 (3)[c]

[a] Contains 1.3% ethanol.
[b] Numbers of experiments are shown in parentheses. Data are mean ± SE.
[c] $p < 0.01$ vs control (*t*-test) and ANOVA shows no significant difference among these three groups.

Table 3
Effect of DES and Estradiol on ATP Hydrolysis by DHP2, DBP2, or DLP2 Fractions

Treatment in different samples	Concentration	F0F1-ATPase activity (%)[a]
DHP2 (15 μg)		
Control	—	100 ± 1.4 (8)
DES	1.3 n*M*	94.2 ± 2.4[b]
	6.7 μ*M*	33.8 ± 1.3[b]
Estradiol	67 μ*M*	88.3 ± 0.9[b]
DBP2 (15 μg)		
Control	—	100 ± 6.2
DES	1.3 n*M*	100 ± 0.9
	67 μ*M*	39.4 ± 1.5[b]
Estradiol	67 μ*M*	80.3 ± 2.3[b]
DLP2 (15 μg)		
Control	—	100 ± 6.6 (5)
DES	1.3 n*M*	101.6 ± 1.6
	6.7 μ*M*	34.4 ± 1.1[b]
Estradiol	67 μ*M*	84.9 ± 2.1[b]

[a] $n = 3$ except for the ones indicated in parentheses.
[b] $p < 0.01$ vs control.
Data are mean ± SE.

Purification of OSCP from DHP2 Through an Estradiol-17β-17-(O-carboxymethyl)oxime: BSA Affinity Column

Since we reported previously that OSCP from a brain preparation was specifically retained by a 17β-E-6-BSA affinity

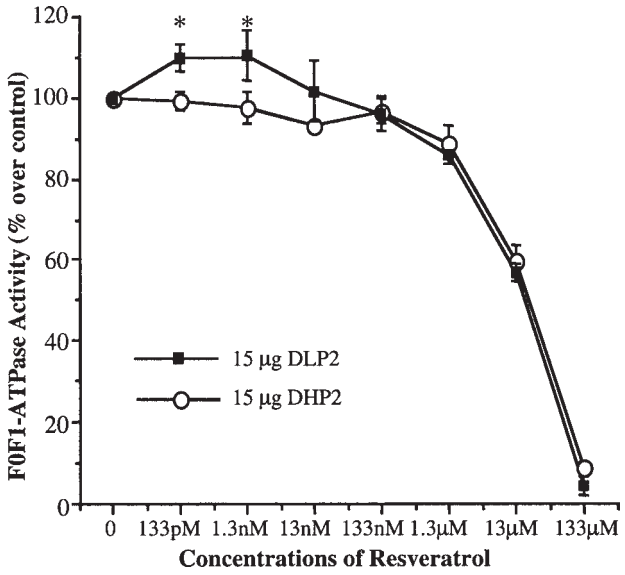

Fig. 8. Effect of resveratrol on F0F1-ATPase activity from DLP2 or DHP2 fractions. Each preparation contained 15 µg of proteins. Resveratrol at micromolar concentrations inhibited the F0F1-ATPase activity in both DLP2 and DHP2. Curiously, 133 p*M* and 1.3 n*M* resveratrol stimulated the F0F1-ATPase activity in DLP2 by about 10% but not in DHP2 ($n = 3$). *$p < 0.05$.

column and showed high affinity for estradiol *(37)*, it was critically important to confirm such findings using a heart preparation. In our study, purification of DHP2 through an estradiol-17β-17-(*O*-carboxymethyl)oxine:BSA (17β-E-17-BSA)-coupled affinity column resulted in a significant retention of a protein of about 28 kDa (Fig. 5). N-terminal protein sequencing of the first 10 amino acids (FSKLVRPPVQ) showed identical N-terminal sequence to mature rat OSCP *(39,40)*. As mentioned previously, the observed molecular size on the gel is different from the calculated molecular size of OSCP. This may owing to a different rate of electrophoresis migration. This protein band is OSCP since it was both recognized specifically by polyclonal anti-OSCP antibody and confirmed by N-terminal protein sequencing. An identical result was observed using a 17β-E-6-BSA-coupled affinity column as well (not shown). Some of the other subunits of the F0F1-ATPase were copurified with OSCP as well, since N-terminal protein sequencing showed that the two strong bands around 52–56 kDa are α- and β-subunits of F0F1-ATPase; the strong band around 34 kDa is γ-subunits of F0F1-ATPase (Zheng and Ramirez, unpublished data). Even though all these proteins were retained by the column, compared with their protein levels before purification (Fig. 5, lane 2), only OSCP showed a significant improvement in protein concentration. Furthermore, when the partially purified fractions were repurified through the same column again, OSCP was the only protein retained (not shown).

Binding of Estradiol-17β-17-(O-carboxymethyl)oxime:^{125}I-labeled BSA to DHP2

Although OSCP was purified from an E-BSA affinity column, one can still argue that the retention was owing to the BSA molecules in the affinity column but not to the estradiol itself. Thus, a displacement assay was performed to determine whether if the binding of the DHP2 to estradiol-17β-17-(*O*-carboxymethyl)oxine:^{125}I-labeled BSA (17β-E-17-[^{125}I]BSA) can be competed by free 17β-estradiol. A low concentration of protein from the heart preparations was used (100 ng/tube) in the assay. At this concentration, about 10% of the total counts per minute added (about 22,000 cpm total, specific activity of 110 µCi/nmol) were bound. Figure 9 shows the average competition curve for three to five different assays. It can be clearly seen that the curve is biphasic, with a "shoulder" at about 10^{-8} *M*, indicating two binding sites. To confirm the number of binding sites predicted from the competition curve, a Scatchard plot was produced and analyzed. Both one- and two-site models were tested for fit. The two-site model showed a better fit as examined by RUNS TEST. As shown in the inset in Fig. 9, two binding components were clearly revealed by Scatchard analysis, one binding site with high affinity of 0.4 n*M* and the other with low affinity of 0.3 µ*M*.

Discussion

In this report we confirmed that the coupled ATPase assay is a valid and sensitive assay to determine the catalytic activity of this ubiquitous multimeric mitochondrial enzyme *(41)*. Low concentrations of protein were sufficient to significantly stimulate ATP hydrolysis within 2 min after adding the digitonin-solubilized mitochondrial preparations to the assay tubes. Inactivation of the enzyme by boiling completely eliminated the activity. Increasing the concentrations of ATP induced higher ATP hydrolysis, indicating that an active ATPase was present in the solubilized preparations from the three different mitochondrial sources studied. The assay conditions lacked Na^+, so the Na^+, K^+-ATPase could not function in this assay. About 84–88% of the ATPase activity in the mitochondrial preparations was from F0F1-ATPase/ATP synthase because it could be inhibited by oligomycin or efrapeptin, or both, confirming similar results published previously *(27,38)*.

Heart samples had a greater catalytic activity than either brain or liver samples. This clear-cut difference in potency, particularly between liver and heart, may have several explanations. First, the mitochondrial preparations from the heart have more quantities of F0F1-ATPase/ATP synthase per microgram of proteins. Second, the intrinsic catalytic activity of the ATPase in the heart is greater than that in the other two tissues. An argument favoring the former is that the gradient in the intensity of the OSCP band detected by Western blotting shows a highest level in the heart, less in

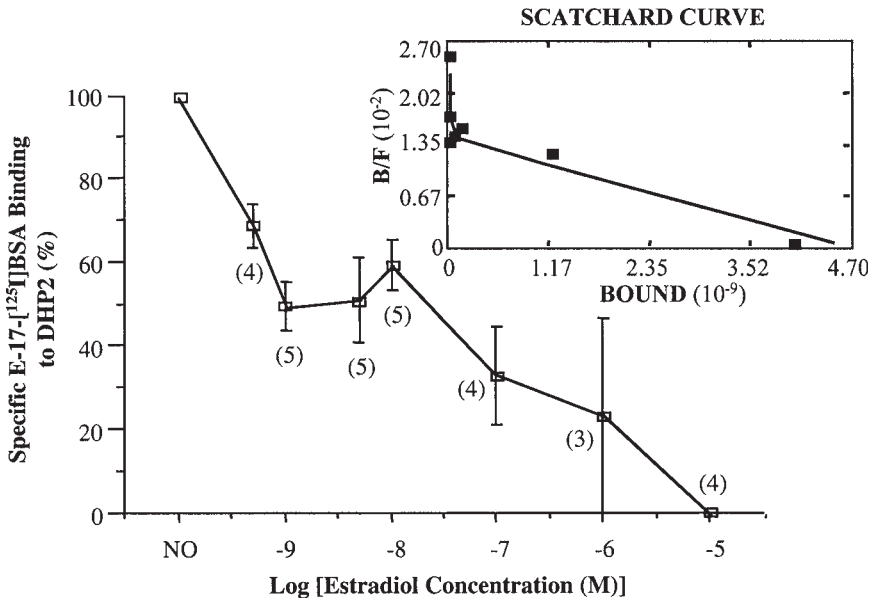

Fig. 9. Displacement of E-17-[^{125}I]BSA by increasing doses of free estradiol. A concentration of 10^{-9} *M* estradiol displaced about 50% of the specific binding of 17β-E-17-[^{125}I]BSA to 0.1 μg of DHP2, whereas 10^{-5} *M* estradiol totally displaced the binding. Binding in the absence of estradiol (NO) was considered 100%. The results revealed a pattern of two estradiol-binding sites in the DHP2 preparation as confirmed by the Scatchard curve (inset). In the Scatchard plot, the *y*-axis represents bound-to-free ligand ratio (B/F), and the *x*-axis represents bound ligand concentration (molar). The K_d values for these two binding sites were 0.4 n*M* and 0.3 μ*M*, respectively, which were calculated using a LIGAND program. The IC_{50} for those two binding sites was about 10^{-9} and 10^{-7} *M*, respectively. Numbers of experiment in duplicates are indicated in parentheses.

the brain and least in the liver. Also, the electron microscopic pictures suggest a greater number of mitochondria in the heart preparation than in the other two samples, with a remarkable difference in the morphology of the mitochondria. In the heart, they are larger and the number of cristae per mitochondrion seems greater than in the other two preparations. However, the OSCP content in the heart is only two- to four-fold higher than that in the brain and liver preparations, which could not totally explain the three- to eight-fold greater activity in the heart as compared with that of the other two preparations. Thus, a difference in the intrinsic ATPase catalytic activity among these three preparations may also exist.

Recently, some other functional differences among three similar mitochondrial preparations were also reported, such as sensitivity to permeability transition *(42)*. The opening of the mitochondrial permeability transition pore (PTP), which is a proteinaceous pore in the inner mitochondrial membrane, is associated with many forms of cell death and several neurogenerative diseases *(42)*. Studies have shown that heart and brain mitochondria are much less sensitive to calcium-induced permeability transition than liver mitochondria *(42–44)*. The extent to which the PTP opens is also much less in brain than liver when dopamine oxidation was used as an inducing factor *(45)*. Interestingly, adenosine 5'-diphosphate (ADP) was found to be able to reverse the opening of PTP or help reseal the permeabilized mitochondria *(46,47)*. Our observation that the ATPase activity differs among these three tissues could be one of the factors that are responsible for their different mitochondrial sensitivity to permeability transition.

The heart and liver preparations, with the highest and lowest specific activity, respectively, were unique in being activated by 17β-estradiol and resveratrol at physiologic concentrations. The activating effect of estradiol worked on the mitochondrial F0F1-ATPase but not on the other contaminating enzymes, because in the presence of efrapeptin the effect was totally eliminated. At pharmacologic concentrations, estradiol was partially inhibitory since a dose of 67 μ*M* inhibited the ATPase from the three preparations by 12%. On the other hand, estradiol, DES (IC_{50} < 7 μ*M*), and resveratrol (IC_{50} of 15 μ*M*), were clearly inhibitory, in agreement with previous results reported by us *(27)* and others *(28)*. The observed effect of the estrogens was on the ATPase but not on PK or LDH as demonstrated previously *(27)*.

The differences among the three estrogenic steroids are intriguing. Although we confirmed in the heart preparation that DES has an inhibitory effect at micromolar concentrations (IC_{50} < 7 μ*M*) similar to the one reported previously by us in the brain *(27)* and others in the liver *(28,48)*, neither this compound nor resveratrol at low concentrations activated the catalytic activity of the enzyme, and only at high doses were they able to inhibit it. Finally, resveratrol did

stimulate the liver preparation at low concentrations and it was clearly inhibitory at micromolar concentrations. If one considers only the low concentrations of the three putative estrogens, we have these results: (1) estradiol stimulates only the heart preparation, (2) resveratrol stimulates only the liver preparation, and (3) DES stimulates none but inhibits the heart preparation. The reason that the same compounds have different actions on the tissues from different origins is still unknown. There are two possible explanations: first, because crude mitochondrial preparations were used, some other proteins in the different preparations may interfere with the interaction between the compounds and the F0F1-ATPase; and, second, it is possible that the intrinsic structure of F0F1-ATPase or its affinity to a specific compound is different, since we have demonstrated significant differences in the enzyme's catalytic activity among the preparations.

Are these differences owing to different sites of interaction in the F0F1-ATPase or to artifacts from the high concentrations of lipophilic substances leading to damage to membrane bilayers and producing nonspecific effects? Apparently, DES requires a defined structure because two hydroxyl groups located at para position on the two benzene rings are crucial as an inhibitor of proton translocation through F0 *(48)*. Other nonestrogenic steroids such as cholesterol, dehydroepiandrosterone, and progesterone, at similar high concentrations, had little effect on the F0F1-ATPase *(27)*. Moreover, physiologic concentrations of estradiol bound to and exerted a specific stimulation of the F0F1-ATPase only in the heart, and resveratrol at low concentrations stimulated the F0F1-ATPase only in the liver, indicating selective but not general nonspecific effects.

The manner in which these selective interactions of these three steroids in the F0F1-ATPase are transmitted to the active sites of catalysis in the F1 sector and the physiologic consequences of such interactions are unknown and worthy of future research. A further study from our laboratory and David Gross's laboratory recently revealed a 20% increase in OSCP concentration in ovariectomized rat heart, while this increase was abolished by 3 mo of estrogen replacement therapy (unpublished findings). Our preliminary results also showed that ovariectomy decreased F0F1-ATPase activity while estrogen replacement restored it. A recent report *(49)* showing that estrogen receptor-α plays a cardioprotective role in ischemia-reperfusion injury in male mice and the fact that ERKO mice revealed clear pathologic changes in the heart mitochondria suggest that estrogen may affect heart functions through changes in ATPase activity.

Since we have reported that E-6-BSA as well as free estradiol bind with high affinity to OSCP, and that an E-6-BSA affinity column selectively retained the F0F1-ATPase from rat brain *(50)*, it was of interest to determine whether the enzyme from mitochondrial preparations of heart would behave similarly. The results confirm our previous reports because the same E-6-BSA conjugate or another E-17-BSA was capable of retaining selectively the F0F1-ATPase from heart. A Western blot demonstrated the presence of OSCP in the purified heart P2, which was confirmed by N-terminal sequencing. In addition, the DHP2 preparation used at a very low concentration of proteins (100 ng/tube) bound 17β-E-17-[^{125}I]BSA with high specific binding (>80%). When free estradiol was used as a competitor, two binding sites were uncovered, one of high affinity (K_d = 0.4 n*M*) and the other of lower affinity (K_d = 300 n*M*). The high-affinity sites most likely correspond to sites in the OSCP subunit of the multimeric enzyme because we reported that a recombinant OSCP from bovine origin bound estradiol with a similar high affinity *(51)*. Therefore, it is possible that this site is responsible for the activation of the enzyme at low and physiologic concentrations of the steroid, whereas the low affinity could be related to the inhibitory effect at pharmacologic concentrations.

It is of interest to speculate about the mechanism of the activation of the enzyme in the heart. ATP synthesis and hydrolysis occurred in the F1 sector, most likely in one of the interfaces between the β- and α-subunits *(52)*, and OSCP appears to be important in channeling the proton gradient from the F0 to F1 sector *(48,53)*. Although we demonstrated that the mitochondrial preparation of heart is rich in OSCP, it is difficult to reconcile this action of OSCP in the solubilized preparations that do not have such a proton gradient, and, therefore, the mechanism has to be different. Because we have reported *(27)* that in an isolated and purified F1 preparation from brain, estradiol did not modify its catalytic activity, it is unlikely that the effect of the steroid occurs in the F1 sector. This reinforces the idea that the effect is related to its binding to OSCP. Since OSCP seems to be part of the stator, estradiol may affect allosterically the catalytic site by changes in conformation of the rotary motor in the enzyme *(54,55)*. These are important areas of question in need of future investigation.

Materials and Methods

Animals

Adult male Sprague-Dawley rats, ages 60–120 d, were kept on a 14:10-h light/dark cycle (lights on at 7:00 AM) with ad libitum access to food and water. Animals were cared for in accordance with federal and institutional guidelines and sacrificed by rapid decapitation.

Preparation of Mitochondrial Fractions (P2 Fractions) from Rat Liver, Brain, and Heart

Pieces of liver tissues, the entire brain, and the ventricle part of heart from adult male Sprague-Dawley rats were dissected out. The heart ventricle was minced into small pieces before further treatment. The samples were homogenized separately (10 up-down strokes [1 min] for liver and brain, 20 up-down strokes [2 min] for heart ventricle) in a Teflon-glass homogenizer in 10 vol of ice-cold GBA buffer

(50 m*M* Tris-HCl, 120 m*M* NaCl, 5 m*M* KCl, 1 m*M* $MgSO_4$, 1 m*M* $CaCl_2$, 10% glycerol, 0.5 m*M* 4-(2-aminoethyl)-benzenesulfonyl fluoride [Boehringer Mannheim, Indianapolis, IN], and 0.1 m*M* bacitracin [Sigma, St. Louis, MO] at pH 7.4 and 4°C). Homogenates were centrifuged at 600*g* for 10 min at 4°C. The pellets (nuclei and cell debris) were removed, and the supernatants were centrifuged again at 14,000*g* for 5 min at 4°C. The resulting supernatants were discarded and the resulting pellets (P2) were resuspended in 200 µL of GBA buffer. The P2 fractions were assayed for protein by the Bradford method using BSA as a reference *(56)*. Finally, the P2 fractions were aliquoted and stored at −80°C for use *(18)*. The P2 pellets mainly consist of mitochondria and lysosomes as determined by electron microscopy analysis *(37)*, which was confirmed in this study.

Solubilization of P2 Fraction by Digitonin

The P2 fractions prepared previously were solubilized in P2-Tris containing 1% digitonin (Sigma; 1 mL of digitonin solution/3 mg of P2 protein) and centrifuged for 60 min at 125,000*g* at 4°C. The supernatants were collected, assayed for protein by the Bradford method, aliquoted, and stored at −80°C until use. Each aliquot of the solubilized P2 fraction was thawed immediately before use, and any remaining volume was discarded after each experiment.

PK/LDH-Coupled ATPase Assay

The coupled assay was based on the method of Harris and Bashford *(41)*. ATPase in the P2 preparations catalyzes ATP hydrolysis to produce ADP and Pi. Its activity was measured spectrophotometrically by coupling the ATP → ADP reaction to an indicator reaction NADH → NAD^+ via the PK and LDH reaction. For every mole of NADH reduced, 1 mol of ATP is hydrolyzed.

Stock solutions of 200 m*M* ATP, 50 m*M* phosphoenol pyruvate, and 100 m*M* NADH (all from Sigma) were prepared in sterile distilled water and stored at −20°C until use. LDH (5000 U/mL) and PK (5000 U/mL) were purchased from Sigma and stored at 4°C until use. Before each experiment, a reaction solution was prepared at room temperature containing 100 m*M* Tris (pH 8.0), 50 m*M* KCl, 0.2 m*M* EDTA, 4 m*M* ATP, 1 m*M* phosphoenol pyruvate, 0.4 m*M* NADH, 2 U/mL of LDH, and 2 U/mL of PK. The concentration of ATP was varied from 0.5 to 8 m*M* when the concentration dependence of ATP on ATP hydrolysis was studied. An appropriate amount of $MgCl_2$ was added into the reaction solution so that the ATP:Mg^{2+} ratio was 1:1. The assay was then started at 30 ± 1°C by adding P2 preparations (5, 15, or 25 µg of proteins) into 0.75 mL of reaction solution. A Hitachi UV/VIS spectrophotometer model U-2001 was programmed to record absorbance at 340 nm *(41)* every 5 s for 5 to 6 min. $\Delta A_{340/min}$ within the linear range was calculated and used to determine initial velocities (V_o) and activity. An extinction coefficient of 6.22 mM^{-1} cm^{-1} was used for NADH. The variation between assays is <3%. When the effect of efrapeptin (kindly provided by Dr. J. Clemens from Eli Lilly, Indianapolis, IN) on ATPase activity was studied, efrapeptin dissolved in sterile distilled water was preincubated with solubilized P2 fractions on ice for 6 min to allow binding *(39)*. When the effect of estradiol, DES, or resveratrol (all from Sigma) on ATPase activity was tested, 10 µL of estradiol, DES, or resveratrol dissolved in ethanol was added into the reaction solution 3 min before the assay was started. The final concentrations of these three compounds ranged from 133 p*M* to 133 µ*M*. For the control experiments, the same amount of vehicle (dH_2O or ethanol) was used instead.

Purification of DHP2 Through 17β-E-17-BSA Affinity Column

17β-E-17-BSA affinity column was prepared by coupling the primary amine groups on the BSA part of 17β-E-17-BSA (2mg) (Sigma) to agarose-activated aldehydes in an AminoLink column (Pierce, Rockford, IL) according to the manufacturer's instructions. Coupling efficiency was about 40–50%. One milliliter of DHP2 (~1 mg of protein/mL) was applied to the affinity column and incubated for 2 h at 4°C. Then another milliliter of DHP2 (~1 mg of protein/mL) was applied and the column was incubated overnight at 4°C. The column was washed with 15 mL of P2-Tris buffer, followed by 5 mL of washing solution (1 *M* NaCl; AminoLink) and 5 mL of 1:10 diluted P2-Tris buffer to remove the unbound proteins. After the wash, the bound proteins were eluted with 0.1 *N* acetic acid (pH 2.7) and collected in 1-mL fractions. Each fraction was assayed for protein concentration by the Bradford method. The protein-containing fractions were pooled and lyophilized. Finally, the lyophilized proteins were resuspended in 1X reducing sample buffer prior to gradient SDS-PAGE.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

DHP2, DBP2, DLP2, or partially purified DHP2 samples were boiled for 5 min in the presence of 1X reducing sample buffer. The samples were centrifuged at 600*g* for about 1 to 2 min. After cooling, the samples were applied to a 4–20% precast polyacrylamide gradient gel (Bio-Rad, Hercules, CA). The electrophoresis was performed for 35 min at 200 V *(57)*. Finally, the gel was stained with FAST stain (Zoion, Allston, MA).

Immunoblotting

The SDS-PAGE was performed as described in the previous section. After electrophoresis, the separated proteins were electrotransferred to a polyvinylidene difluoride (PVDF) membrane (Applied Biosystems, Foster City, CA) at 50 V and 4°C for 2 h *(58)*. The membrane was then blocked with 1% BSA in phosphate-buffered saline (PBS) at 37°C for 1.5 h and washed three times with PBS. Polyclonal rabbit anti-bovine OSCP antibody was kindly provided by Dr. G. I. Belogrudov, Dr. A. Matsuno-Yagi, and Dr. Y. Hatefi from

Scripps Research Institute, La Jolla, CA. The blot was incubated with the antibovine OSCP antibody (1:1000 dilution in PBS with 1% BSA) for 1.5 h at room temperature and then washed three times with PBS. The second antibody used was goat antirabbit IgG antibody conjugated with horseradish peroxidase (Sigma). The blot was incubated with the second antibody (1:1000 dilution in PBS with 1% BSA) for 1 h at room temperature and then washed with PBS three times. Immunoreactive proteins were visualized using a diaminobenzidine substrate system with $NiCl_2$ (0.05%; both from Sigma) enhancement. The relative intensities of the immunoreactive proteins were analyzed using the NIH IMAGE program after the membrane was scanned and the image was imported into the computer. The intensity of the immunoreactive protein from DHP2 and DBP2 was compared with that from DLP2.

N-Terminal Sequencing

Partially purified proteins from DHP2 were separated by SDS-PAGE and then electrotransferred to a PVDF membrane in a Tris-glycine-based transfer buffer at 50 V and 4°C for 45 min *(59)*. After washing with dH_2O for 30 min, the membrane was stained with 0.1% Coomassie brilliant blue (Sigma) R-250 and destained with 50% methanol. The membrane was washed for 2 h with dH_2O to rinse off the extra glycine on the membrane. After air-drying, the bands of interest were excised. N-terminal amino acids were sequenced on an Applied Biosystems Model 577A sequencer coupled to a Model 120A on-line PTH analyzer using Edman chemistry.

Electron Microscopy

P2 fractions from rat liver, brain, and heart were pelleted by centrifugating at 600*g* for 10 min. The fractions were fixed in Karnovsky's fixative overnight at 4°C. The samples were then embedded in LX-112 epoxy, and the epoxy was polymerized for 2 d at 70°C. The resulting blocks were trimmed and sectioned at 50–70 nm, and the sections were mounted on copper grids. The sections were examined with a Hitachi H-600 electron microscope at different magnifications in the Center for Microscopy and Imaging of the University of Illinois at Urbana-Champaign.

Binding Assay

The binding assay was based on the method of Ramirez and Zheng *(20)*. Briefly, 17β-E-17-[^{125}I]BSA (22,000 cpm, specific activity of 110 μCi/nmol) was prepared as described *(37)*. Appropriate amounts of Tris binding buffer (50 m*M* Tis-HCl, 120 m*M* NaCl, 5 m*M* KCl, 1 m*M* $MgSO_4$, 1 m*M* $CaCl_2$, 10% glycerol, and 0.08% BSA at pH 7.4 at 4°C), unlabeled competitor (estradiol), and labeled ligand (17β-E-17-[^{125}I]BSA) were added to each tube in duplicates. The DHP2 fractions (0.1–0.5 μg of protein) were added and the tubes were shaken for 30 min at 4°C. The reaction was stopped by adding 4 mL of binding buffer to each tube and rapidly filtering under vacuum through a Whatman GF/C glass fiber filter using a Model 1225 sampling manifold. This procedure was repeated once. Finally, each filter was removed and placed in test tubes, and the radioactivity of the ligand retained on the filter was counted using a gamma counter (70 ± 2% efficiency, $n = 10$). Specific binding was considered as Total Binding – Nonspecific Binding (NSB). NSB corresponds to competition by 10^{-5} *M* estradiol. Scatchard plots *(60)* were produced using the mean values of the assays and analyzed by an NIH LIGAND program *(61)*. The goodness of fit for Scatchard plot was estimated by using the RUNS TEST *(61)*. Dissociation constant (K_d) values were calculated using the LIGAND program.

Statistical Analyses

When $n \geq 3$, means ± SE are given. Unless specifically clarified, n represents the number of assays tested using n batches of tissue preparations. One- and two-way ANOVA followed by a Tukey post-hoc analysis were used for statistical comparisons among groups ($n = 6$ or 7 per group). A value of $p < 0.05$ was considered significant.

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References

1. Hendry, L. B. and Mahesh, V. B. (1995). *J. Steroid. Biochem. Mol. Biol.* **55,** 173–183.
2. Evans, R. M. (1988). *Science* **240,** 889–895.
3. Beato, M. (1989). *Cell* **56,** 335–344.
4. Groner, B., Hynes, N. E., Rahmsdorf, J., and Ponta, H. (1983). *Nucleic Acids Res.* **11,** 4713–4725.
5. Cato, A. C. B. and Weinmann, J. (1988). *J. Cell Biol.* **106,** 2119–2125.
6. Lieberherr, M. and Grosse, B. (1994). *J. Biol. Chem.* **269,** 7217–7223.
7. Wasserman, W. J., Pinto, L. H., O'Connor, C. M., and Smith, L. D. (1980). *Proc. Natl. Acad. Sci. USA* **77,** 1534–1536.
8. Gekle, M., Silbernagl, S., and Oberleithner, H. (1997). *Am. J. Physiol.* **273,** C1673–C1678.
9. Farhat, M. Y., Abi-Younes, S., Dingaan, B., Vargas, R., and Ramwell, P. W. (1996). *J. Pharmacol. Exp. Ther.* **276,** 652–657.
10. Wehling, M. (1997). *Annu. Rev. Physiol.* **59,** 365–393.
11. Schwartz, Z., Gates, P. A., Nasatzky, E., Sylvia, V. L., Mendez, J., Dean, D. D., and Boyan, B. D. (1996). *Biochim. Biophys. Acta* **1282,** 1–10.
12. Endoh, H., Sasaki, H., Maruyama, K., Takeyama, K., Waga, I., Shimizu, T., Kato, S., and Kawashima, H. (1997). *Biochem. Biophys. Res. Commun.* **235,** 99–102.
13. Wong, M. and Moss, R. L. (1991). *Brain Res.* **543,** 148–152.
14. Berg, B. H. (1997). *Biochim. Biophys. Acta* **479,** 152–171.

15. Mermelstein, P. G., Becker, J. B., and Surmeier, D. J. (1996). *J. Neurosci.* **16,** 595–604.
16. Audy, M. C., Vacher, P., and Dufy, B. (1996). *Eur. J. Endocrinol.* **135,** 367–373.
17. Gu, Q. and Moss, R. L. (1996). *J. Neurosci.* **16,** 3620–3629.
18. Ramirez, V. D. (1992). *Neuroprotocols: Companion Methods Neurosci.* **1,** 35–41.
19. Ke, F. C. and Ramirez, V. D. (1990). *J. Neurochem.* **54,** 467–472.
20. Ramirez, V. D. and Zheng, J. (1996). *Front. Neuroendocrinol.* **17,** 402–439.
21. Moats, R. K. and Ramirez, V. D. (1998). *Biol. Reprod.* **58,** 531–538.
22. Moats, R. K. and Ramirez, V. D. (2000). *J. Endocrinol.* **166,** 631–647.
23. Ramirez, V. D. and Zheng, J. (1996). *Cell. Mol. Neurobiol.* **16,** 175–198.
24. Voet, D. and Voet, J. G,, eds. (1990). *Biochemistry.* John Wiley & Sons: New York.
25. Stryer, L., ed. (1995). *Biochemistry,* 4th ed. W. H. Freeman: New York.
26. Wang, H. and Oster, G. (1998). *Nature* **396,** 279–282.
27. Zheng, J. and Ramirez, V. D. (1999). *Eur. J. Pharmacol.* **368,** 95–102.
28. McEnery, M. W. and Pedersen, P. L. (1986). *J. Biol. Chem.* **261,** 1745–1752.
29. Gehm, B. D., McAndrews, J. M., Chien, P. Y., and Jameson, J. L. (1997). *Proc. Natl. Acad. Sci. USA* **94,** 14,138–14,143.
30. Mizutani, K., Ikeda, K., Kawai, Y., and Yamori, Y. (1998). *Biochem. Biophys. Res. Commun.* **253,** 859–863.
31. Stahl, S., Chun, T. Y., and Gray, W. G. (1998). *Toxicol. Appl. Pharmacol.* **152,** 41–48.
32. Humfrey, C. D. (1998). *Nat. Toxins* **6,** 51–59.
33. Strauss, L., Santti, R., Saarinen, N., Streng, T., Joshi, S., and Makela, S. (1998). *Toxicol. Lett.* **102–103,** 349–354.
34. Lardy, H. A., Johnson, D., and McMurray, W. C. (1958). *Arch. Biochem. Biophys.* **78,** 587–597.
35. Cross, R. L. and Kohlbrenner, W. E. (1978). *J. Biol. Chem.* **253,** 4865–4873.
36. Higuti, T., Kuroiwa, K., Kawamura, Y., Morimoto, K., and Tsujita, H. (1993). *Biochim. Biophys. Acta.* **1172,** 311–314.
37. Zheng, J. and Ramirez, V. D. (1997). *J. Steroid Biochem. Mol. Biol.* **62,** 327–336.
38. Zheng, J. and Ramirez, V. D. (1999). *Biochem. Biophys. Res. Commun.* **261,** 499–503.
39. Cretin, F., Baggetto, L. G., Denoroy, L., and Godinot, C. (1991). *Biochem. Biophys. Acta* **1058,** 141–146.
40. Higuti, T., Kuroiwa, K., Kawamura, Y., Morimoto, K., and Tsujita, H. (1993). *Biochem. Biophys. Acta* **1172,** 311–314.
41. Harris, D. A. and Bashford, C. L., eds. (1987). *Spectrophotometry and spectrofluorimetry: a practical approach.* IRL: Oxford.
42. Berman, S. B., Watkins, S. C., and Hastings, T. G. (2000). *Exp. Neurol.* **164,** 415–425.
43. Palmer, J. W. and Pfeiffer, D. R. (1981). *J. Biol. Chem.* **256,** 6742–6750.
44. Novgorodov, S. A., Gudz, T. I., Milfrom, Y. M., and Brierley, G. P. (1992). *J. Biol. Chem.* **267,** 16,274–16,282.
45. Berman, S. B. and Hastings, T. G. (1999). *J. Neurochem.* **73,** 1127–1137.
46. Haworth, R. A. and Hunter, D. R. (1980). *J. Membr. Biol.* **54,** 231–236.
47. Crompton, M. and Costi, A. (1990). *Biochem. J.* **266,** 33–39.
48. McEnery, M. W., Hullihen, J., and Pedersen, P. L. (1989). *J. Biol. Chem.* **264,** 12,029–12,036.
49. Zhai, P. Y., Eurell, T. E., Cooke, P. S., Lubahn, D. B., and Gross, D. R. (2000). *Am. J. Physiol. Circ. Physiol.* **278,** H1640–H1647.
50. Zheng, J. and Ramirez, V. D. (1999). *J. Steroid Biochem. Mol. Biol.* **68,** 65–75.
51. Ramirez, V. D. and Zheng, J. (1999). In: *Estrogens and antiestrogens I.* Oettel, M. and Schillinger, E. (eds.). Springer-Ver-lag: Berlin.
52. Stock, D., Leslie, A. G. W., and Walker, J. E. (1999). *Science* **284,** 1700–1705.
53. Boyer, P. D. (1997). *Annu. Rev. Biochem.* **66,** 717–749.
54. Kaim, G. and Dimroth, P. (1999). *EMBO J.* **18,** 4118–4127.
55. Pedersen, P. L. (1999). *J. Bioenerg. Bio.* **31,** 291–304.
56. Bradford, M. M. (1976). *Anal. Biochem.* **72,** 248–254.
57. Laemmli, U. K. (1970). *Nature* **227,** 680–685.
58. Towbin, H., Staehelin, R., and Gordon, J. (1979). *Proc. Natl. Acad. Sci. USA* **76,** 4350–4354.
59. Matsudaira, P. (1987). *J. Biol. Chem.* **261,** 10,035–10,038.
60. Paál, K., Müller J., and Hegedûs, L. (2001). *Eur. J. Biochem.* **268,** 2187–2191.
61. Munson, P. J. and Rodbard, D. (1980). *Anal. Biochem.* **107,** 220–239.