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Binding of estrogen and progesterone-BSA conjugates to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the effects of the free steroids on GAPDH enzyme activity: physiological implications

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Abstract

In this study rat brain solubilized plasmalemma-microsomal fractions (B-P3) or cytosolic fractions were applied to P-3-BSA (progesterone linked to BSA at C-3 position) and E-6-BSA (17β-estradiol linked to BSA at C-6 position) affinity columns. It is interesting that a 37 kDa protein was retained by both columns which was identified as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by N-terminal sequencing. The 37 kDa protein (GAPDH) was not retained by either a control BSA conjugated affinity column or a corticosterone-BSA affinity column. E-6-BSA bound to GAPDH with higher binding affinity than P-3-BSA or T-3-BSA (testosterone linked to BSA at C-3 position) affinity columns. In addition, the binding of 17β-E-6-BSA to GAPDH was impeded by free estrogen (17β-estradiol) completely. Binding studies of E-6-BSA and P-3-BSA to commercial GAPDH from rabbit skeletal muscle using radiolabeled ligand binding assays revealed that P-3-BSA had 10× lower GAPDH binding affinity than E-6-BSA. Next, the effects of estrogen and progesterone on GAPDH activity were studied. Rapid and significant increases in V_{max} and changes in K_m were observed by the addition of 10 nM estradiol, whereas 100 nM progesterone decreased only V_{max} significantly. Testosterone, corticosterone, 17α-estradiol, and diethylstilbestrol did not affect the enzyme activity. The results indicate that GAPDH is a target site for 17β-estradiol and progesterone and suggest possible roles in the regulation of cellular metabolism and synaptic remodeling in which GAPDH has been reported to be involved.

Keywords: 17β-Estradiol; Progesterone; Glyceraldehyde-3-phosphate dehydrogenase (GAPDH); Affinity chromatography; Enzyme activity

1. Introduction

It has been well established that the major mechanism of action of sex steroid hormones, such as estrogen and progesterone, involves intracellular receptor proteins. The hormone/receptor complexes function as transcription factors which can up-regulate or down-regulate a particular gene expression [1,2]. However, there is evidence of non-conventional mechanisms of action of steroid hormones in different types of tissues. One of the characteristics of the so called 'non-genomic' actions of steroid hormones is the binding of steroid hormones to membrane bound proteins or the induction of rapid changes in plasma membrane properties [3,4]. Proteins with high-binding affinity to progesterone have been found in several different types of tissues or cells such as mouse brain tissue, xenopus oocytes, porcine liver tissue and human sperms [5–8]. Progesterone caused a rapid increase in intracellular calcium concentration within 40–60 s in amphibian oocytes and rapid/reversible reduction in the contractile force of the smooth muscle [9,10]. This decrease in the contractile force of smooth muscles caused by progesterone could not be blocked by progesterone receptor inhibitors [10]. Similarly, estrogen has been shown to increase protein kinase C activity in the resting zone and growth zone of chondrocytes within 10 min, implying alternative signal mechanisms possibly mediated by a putative membrane associated estrogen receptor [11]. In neural tissue the increase of the activity of MAP-kinase and tyrosine kinase by estrogen within 30 min was

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independent of gene transcription or protein synthesis [12]. This result suggests the involvement of non-genomic action of estrogen in the protection of neural cells against glutamate toxicity.

Besides the criteria of non-genomic actions of steroid hormones described above, there is another alternative for the non-genomic effect of steroids. Earlier it was shown that steroid hormones can interact with several enzymes which do not function as direct regulators of gene expressions, such as aldehyde dehydrogenase and glucose-6-phosphate dehydrogenase [13]. These early studies were considered as a potential model of the mechanism of steroid action. A recent renewed effort in this direction is worth mentioning. For example, estrogen increases the activity of 17-aminoacyl-tRNA synthetase in rat uterus [14] and the autophosphorylation activity of ErbB2 tyrosine kinase by binding to the extracellular ligand binding domain of ErbB2 proteins with a Kd of about 2.7 nM [15]. Hayashi et al. recently reported that the activity of neuronal nitric oxide synthase was affected by estradiol in a biphasic manner: nanomolar concentrations activated the enzyme, whereas micromolar concentrations attenuated its activity [16]. The activation of adenylate cyclase by estrogen is another example [17].

Previously, in our laboratory the interaction of estrogen with membrane binding proteins in the rat brain and liver has been demonstrated. Using estrogen-BSA conjugated affinity chromatography, Zheng and Ramirez found that oligomycin sensitive-conferring protein (OSCP), a subunit of proton F0F1-ATPase/ATP synthase, in synaptosomal fractions of female rat brains binds to estradiol [18]. Subsequently, they demonstrated that the activity of the mitochondrial protein, F0F1-ATPase, in rat brain was inhibited rapidly by estrogen [19]. Moats and Ramirez have shown two estrogen binding sites in the plasmalemma microsomal fractions of liver and rapid translocation of radiolabeled estrogen-BSA conjugates from plasma membrane to mitochondria and lysosome, implying a potential role of membrane estrogen-binding proteins in rapid endocytosis of estrogen [20]. Similarly, our laboratory has studied the interaction of progesterone with membrane binding proteins in the rat brain. The cerebral cortex, brain stem, cerebellum, corpus striatum and hypothalamus of female rat brains were shown to have high binding affinity sites to progesterone [21]. A 40–50 kDa protein having high binding affinity to progesterone was purified from cerebellum of rat brains using affinity chromatography [22].

In this study we describe the isolation and affinity purification of a 37 kDa protein from plasmalemma microsomal fractions of rat brains by both 17β-estradiol-BSA and progesterone-BSA affinity columns. N-terminal sequence of the protein showed 100% homology to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). We also studied the binding of GAPDH to other affinity columns, specifically T-3-BSA, C-21-BSA, and BSA. In addition, in a binding assay commercial GAPDH bound 17β-E-6-BSA with high affinity, whereas progesterone-BSA was a poor competitor. Furthermore, we investigated the effects of progesterone and estradiol on the enzyme activity of GAPDH and compared to other steroids, such as testosterone, corticosterone, diethylstilbestrol and 17α-estradiol.

2. Experimental

2.1. Animals

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

2.2. Preparation of membrane fraction

The plasmalemma microsomal fraction of rat brain (B-P3) was prepared as follows. The brains of adult female rats (undisclosed estrous cycle) were dissected, placed in 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) and homogenized in 10× vol. of P2-Tris Buffer with 0.5 mM AEBSF and 0.1 mM bacitracin. The homogenate was centrifuged at 4°C, 600 × *g* for 10 min. The supernatant fraction (S1) was then centrifuged at 4°C, 15 000 × *g* for 5 min to obtain a second supernatant (S2). This supernatant (S2) underwent ultra-centrifugation at 4°C, 125 000 × *g* for 90 min. The pellet (B-P3) and supernatant (S3) (plasmalemma microsomal fraction and cytosolic fraction, respectively) were collected, aliquots were prepared and stored at −70°C. Protein concentration was measured by the Bradford method using BSA as a reference [23].

2.3. Solubilization of membrane fraction

B-P3 fractions were diluted with distilled water to 1:1 followed by the addition of Triton X 100 (1% Triton X 100 in final volume). After stirring for 1 h at 0°C the sample was centrifuged at 125 000 × *g* for 75 min at 4°C. The supernatant (solubilized membrane fraction) was collected and frozen at −70°C. Protein concentration was measured by the Bradford method using BSA as a reference [23].

2.4. Affinity purification of 37 kDa protein

17β-E-6-BSA and P-3-BSA affinity columns were prepared by linking the primary amine groups in the BSA part of the steroid-BSA conjugate to agarose activated aldehydes in an AminoLink column (Pierce) as described before [22] and stored at 4°C. Solubilized membrane fractions of rat brain were applied to 17β-E-6-BSA and P-3-BSA columns (1 ml of sample at a time) and incubated for 2 h at 4°C. A total of 4 ml (8–9 mg) of sample was applied. The column was then washed with 15–20 ml P2-Tris buffer to remove

the unbound proteins, followed by washing with 4–5 ml 5 mM Tris·HCl (pH 7.4) plus 1% glycerol, 1 mM $CaCl_2$ and 1 mM $MgSO_4$. The bound proteins were eluted with 12 × 1ml 0.1 N acetic acid (pH 2.7). For the repurification purpose the elutes were neutralized with 1 M Tris pH 9.4 followed by the addition of these elutes into the column, and the steps described above were repeated. The protein concentration was measured by the Bradford method [23]. The eluted fractions were pooled and lyophilized for SDS-PAGE analysis.

2.5. SDS-PAGE

The lyophilized samples were suspended with 1 × reducing sample buffer and heated for 5 min at 95°C. The samples were applied to 4–20% precast polyacrylamide gradient gel (Bio-Rad) and electrophoresis was performed at 200 V for 32 min followed either by staining with Fast stain (0.1% Coomassie Brilliant Blue) or by blotting on PVDF membrane.

2.6. N-terminal sequencing

The purified solubilized membrane fractions separated by SDS-PAGE were transferred to PVDF membrane in the presence of reduced glutathione at 50 V for 45 min. The membranes were stained with 0.1% Coomassie Brilliant Blue R-250. N-terminal amino acids were sequenced on an Applied Biosystems model 577A sequencer coupled to a model 120A online PTH analyzer using Edman chemistry.

2.7. Western blot

Proteins separated by SDS-PAGE were transferred to PVDF membrane in the absence of glutathione for 2 h at 50 V. The membranes were incubated with blocking agent (1% BSA-PBS) for 1.5 h at 28°C or overnight at 4°C, followed by washing with PBS three times (5 min each). The blot incubated with monoclonal GAPDH antibody (1:100, Biodesign International) in 1% BSA-PBS for 1.5 h at 28°C was washed with PBS three times (each 5 min), and it was incubated for 1 h at room temperature with goat anti-mouse IgG secondary antibody conjugated to horse-radish peroxidase (1:5000, Chemicon). After washing, the blot was developed by using DAB enzyme substrate.

2.8. 17β-E-6-[^{125}I]-BSA binding assay

Radioiodination of 17β-E-6-BSA and the radioligand binding assay were performed as described before [18,21] except that 4 μg (54 nM in final volume of 500 μl) of synthetic rabbit muscle GAPDH (Sigma) and GF/F filter (glass microfiber filters, Whatman) were employed. Briefly, the enzyme and radiolabeled ligand (45 000 cpm/500 μl, 0.12 nM) were mixed in 0.08% BSA-P2 Tris buffer in the presence of unlabeled ligands. The solution was incubated for 30 min at 4°C with shaking. The bound radiolabeled ligand remained on the filter paper in a mannifold. The radioactivity of filter paper was counted using a gamma counter for 1 min with 70% efficiency.

2.9. 17β-E-6-BSA affinity chromatography of commercial GAPDH in the absence and presence of free 17β-estradiol

GAPDH (2.5 μg) was resuspended in P2-Tris buffer at pH 7.0 followed by addition of either 10 μl ethanol (control) or 10 μl of 0.1 mM 17β-estradiol. The total volume was 1 ml. After the mixture was incubated with shaking at 4°C for 30 min, the solution was applied to the 17β-E-6-BSA affinity column. Incubation of the column and elution were performed as described above. The elutes were analyzed by SDS-PAGE.

2.10. GAPDH activity assay

The enzymatic activity of GAPDH was measured by a slightly modified method described by Kant and Steck [24]. 1 μg of synthetic GAPDH (Sigma) suspended in distilled water was added to sodium pyrophosphate buffer (pH 7, 0.5 ml), followed by the addition of a certain concentration of 17β-estradiol (in 0.01 ml) and 0.05 ml of β-NAD (20 mM). A final concentration of 1.4% ethanol (0.01 ml of 95% ethanol) was added to control groups. The reaction was initiated by adding 0.12 ml of mixture of sodium arsenate (0.4 M) and glyceraldehyde-3-phosphate (15 mM, adjusted to pH 7.0) in a total volume of 0.68 ml. In enzyme kinetic assays, different concentrations of G-3-P were used. Taking the addition of glyceraldehyde-3-phosphate as the starting point of the reaction, absorbance and rate were continuously measured spectrophotometrically (Hitachi U-2001) at 340 nm and at 28–30°C.

2.11. Statistical analysis

The data are expressed as a mean ± SE (standard error) and analyzed by the analysis of variance (ANOVA) between control and experimental groups. $P < 0.05$ was considered significant.

3. Results

As expected SDS-PAGE analysis of the crude solubilized plasma membrane-microsomal fraction (P3) and cytosolic fraction showed a great number of protein bands (data not shown). P-3-BSA affinity chromatography shows about 6–8 major P-3-BSA conjugate binding proteins (Fig. 1, lane 2). Repurification resulted in three major proteins, 37 kDa, 28 kDa, and 20 kDa proteins that were well retained (Fig. 1, lane 3). The 37 kDa protein was also purified from cytosolic fractions or from plasma membrane-microsomal

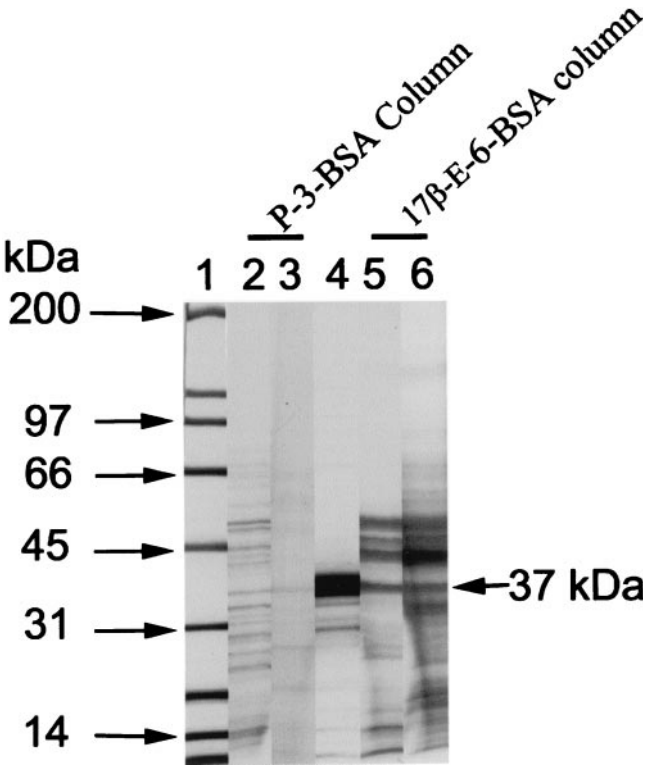

Fig. 1. SDS-PAGE analysis of affinity purified samples from rat brains by using estrogen-/progesterone affinity columns. **Lane 1**: molecular weight standard (6 μg loaded in the gel), **Lane 2**: P-3-BSA affinity purified solubilized plasma membrane-microsomal sample of rat brains (6 μg), **Lane 3**: P-3-BSA affinity re-purified solubilized plasma membrane-microsomal sample of rat brains (approximately 0.3 μg), **Lane 4**: rabbit muscle GAPDH (1.6 μg), **Lane 5**: 17β-E-6-BSA affinity purified cytosolic sample of rat brains (10 μg), **Lane 6**: 17β-E-6-BSA affinity purified solubilized plasma membrane-microsomal sample of rat brains (10 μg). GAPDH; glyceraldehyde-3-phosphate dehydrogenase, P-3-BSA; progesterone-3-(O-carboxylmethyl)oxime-BSA conjugate, 17β-E-6-BSA; 17β-estradiol 6-(O-carboxymethyl)oxime-BSA conjugate.

A.

NH2-**Val-Lys-Val-Gly-Val-Asn-Gly-Phe-Gly-Arg-Ile-Gly-Arg-Leu-Val-Thr-Arg-Ala-?-Phe**-COOH

B.

37 kDa protein	**VKVGVNGFGRIGRLVTRA?F**----------
Rat GAPDH	**VKVGVNGFGRIGRLVTRAAF**SCDKVDI-------
Mouse GAPDH	**VKVGVNGFGRIGRLVTRAAI**CSGKVEIV------
Rabbit GAPDH	**VKVGVNGFGRIGRLVTRAAF**NSGKVDV------
Human GAPDH	**VKVGV*DGFGRIGRLVTRAAF**NSGKVDIV---

Fig. 2. N-terminal sequence of the 37 kDa protein (A) and comparison with other species (B). A. Identity of the twenty amino acid residues from the purified 37 kDa protein. The 19th amino acid (?) of the purified 37 kDa protein was ambiguous. B. Comparison of the sequences from the purified 37 kDa protein with other GAPDH sequences from other species. The underline indicates identical amino acids residues among GAPDHs from different species. The purified 37 kDa protein showed 100% homology to GAPDH of the different species.

(P3) fractions of female rat brains (Fig. 1, lane 5 and 6, respectively) by using 17β-E-6-BSA affinity chromatography. It is interesting that although approximately 60% more proteins were eluted from the 17β-E-6-BSA affinity column than those from P-3-BSA affinity column (Fig. 1, lane 6 and lane 2, respectively), the intensity of the purified 37 kDa protein band from the 17β-E-6-BSA column was much stronger (at least 5 times more compared to the band from the elutes of the P-3-BSA affinity column) suggesting higher binding affinity of the 37 kDa protein to 17β-E-6-BSA than to P-3-BSA.

The 37 kDa protein (cytosolic and P3 fraction origin) was identified as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by N-terminal amino acid sequencing. The first 20 amino acid residues of the 37 kDa protein were resolved (Fig. 2A). The 19th amino acid was not clearly determined. The identified amino acid residues were 100% homologous to GAPDH of rat, mouse, rabbit, and human (Fig. 2B). Only one amino acid of human GAPDH did not match with the corresponding 6th amino acid (*) of the 37 kDa protein, indicating that the amino acid sequences of the 37 kDa protein are conserved in high degree among the species. Since GAPDH is a well known cytosolic glycolytic enzyme and was purified from both the cytosolic and P3 fraction, it was speculated that the latter fraction (P3) could be contaminated by cytosolic proteins during subcellular centrifugation. Thus, we attempted to wash the P3 fraction further by using ultra-centrifugation (128 000 $\times$ g, for 30 min). Fig. 3 shows that the 37 kDa protein (GAPDH) band is barely detectable after an extra washing step (lane 4). The same observation was also seen in Western blot analysis (data not shown). This result led us to think of two possibilities; the first is contamination of the P3 fraction by cytosolic proteins and the second is that the binding of GAPDH to plasma/microsomal membrane is too weak to be maintained after an extra washing step using such high speed centrifugation.

Regardless of the origin of the purified GAPDH, it was considered of interest to study the binding specificity of 17β-E-6-BSA to GAPDH compared to other steroid-BSA conjugates (Fig. 4). The same amount of commercial GAPDH (20 μg) was applied to all five different steroid-BSA conjugates affinity columns, specifically the 17β-E-6-

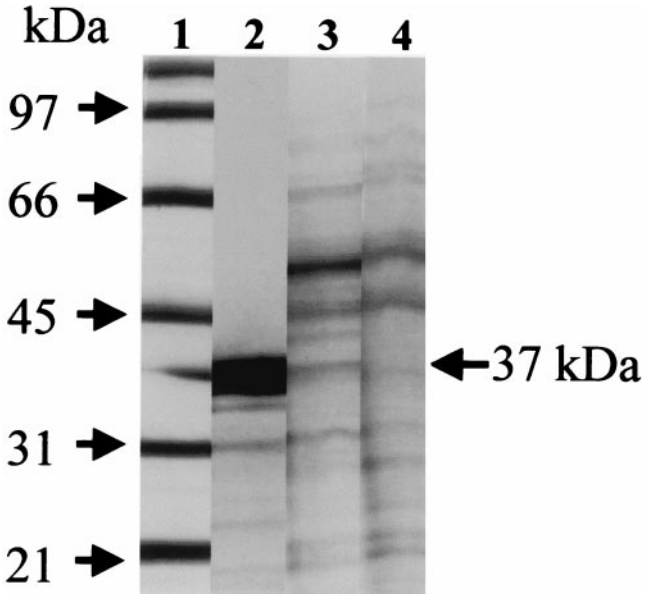

Fig. 3. The intensity of the 37 kDa protein band after extra washing by centrifugation at 128 000 × *g*, 4°C for 30 min. **Lane 1**: molecular weight standard (10 μg), **Lane 2**: rabbit muscle GAPDH (2.6 μg), **Lane 3**: 17β-E-6-BSA affinity purified solubilized plasma membrane-microsomal sample of rat brains (14.6 μg), **Lane 4**: 17β-E-6-BSA affinity purified solubilized plasma membrane-microsomal sample of rat brains after one more washing step (14.6 μg).

BSA, P-3-BSA, T-3-BSA, C-21-BSA, and BSA affinity columns (Fig. 5A). As we expected, the 17β-E-6-BSA and P-3-BSA affinity columns retained GAPDH (Fig. 5A, lanes 1 and 2). Interestingly, the T-3-BSA affinity column also retained GAPDH (Fig. 5A, lane 3), whereas the C-21-BSA and BSA affinity columns did not retain the enzyme (Fig. 5A, lanes 4 and 5). Among the three steroid-BSA conjugate columns, 17β-E-6-BSA affinity column showed the highest binding affinity to GAPDH, which agrees with our previous findings (Fig. 1).

Besides affinity chromatography we further studied the binding of estrogen-BSA and progesterone-BSA to GAPDH by using a competitive binding assay. In this assay 17β-E-6-[^{125}]I-BSA was used as a radiolabeled ligand, and 17β-E-6-BSA and P-3-BSA were used as unlabeled competitors. Also, native rabbit muscle GAPDH was employed since it was identical to the 37 kDa protein. The binding of the radiolabeled ligand to GAPDH (4 μg/0.5 ml, 54 nM) was about 10% of the total cpm in the reaction solution (total 45 000 cpm in 0.5 ml, 0.12 nM). The molar concentration of GAPDH was based upon the molecular weight of tetrameric GAPDH (molecular weight of a subunit is 37 kDa). 50 nM of unlabeled 17β-E-6-BSA displaced 50% of the radiolabeled ligand bound to GAPDH, whereas 500 nM of unlabeled P-3-BSA was needed to do so (Fig. 5B). This indicates that 17β-E-6-BSA had 10 × higher binding affinity to GAPDH than P-3-BSA. In addition, Fig. 5C shows that not

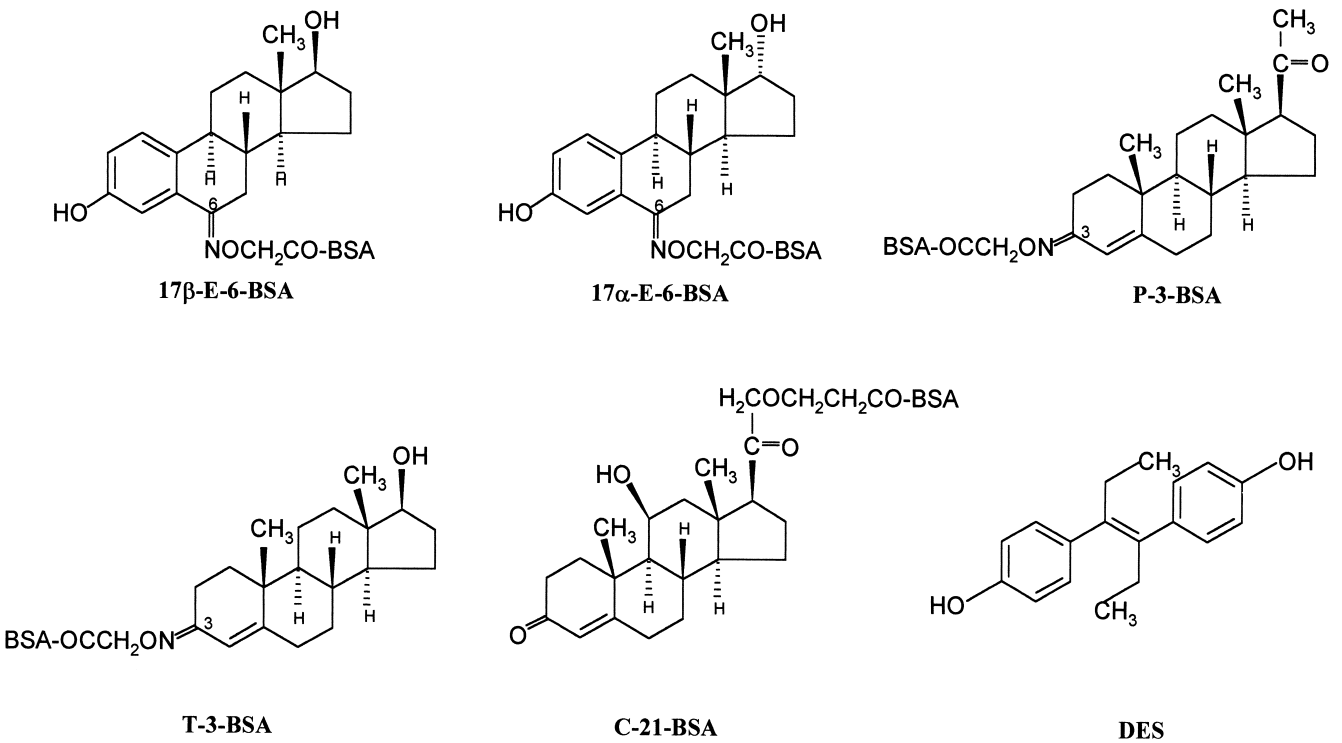

Fig. 4. Planar structures of the steroid-BSA complexes and diethylstilbestrol (DES) used in these experiments. 17β-E-6-BSA; 17β-estradiol 6-(O-carboxymethyl)oxime-BSA conjugate (32 moles 17β-estradiol/BSA), P-3-BSA; progesterone-3-(O-carboxymethyl)oxime-BSA conjugate (20 moles progesterone/BSA), T-3-BSA; testosterone-3-(O-carboxymethyl)oxime-BSA conjugate (29 moles/BSA), C-21-BSA; corticosterone-21-hemisuccinate-BSA conjugate (23 moles corticosterone/BSA).

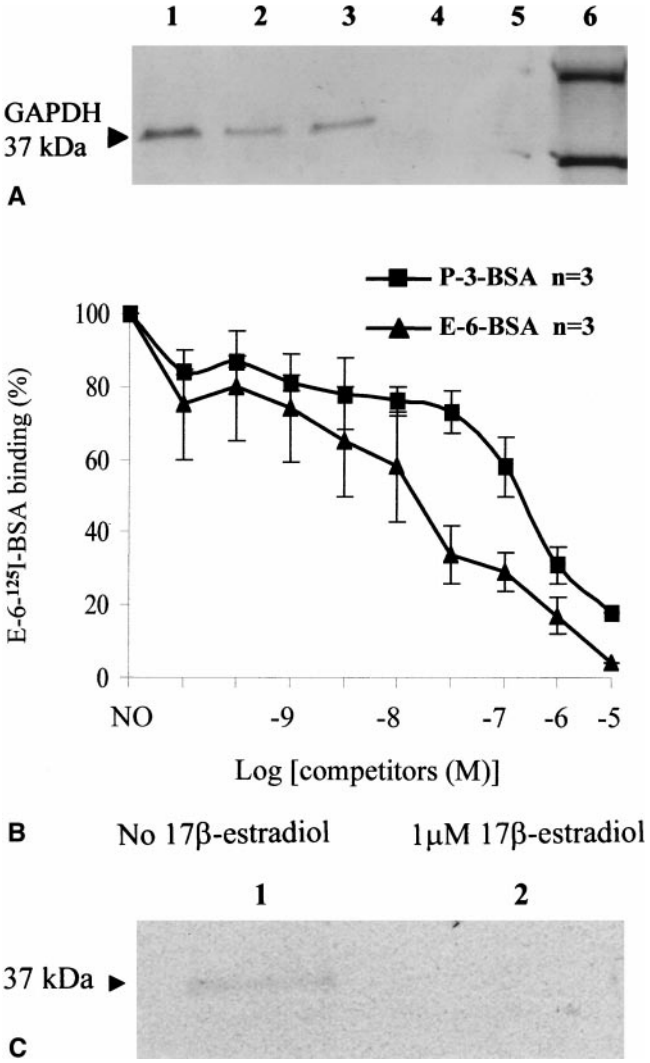

Fig. 5. A. 20 μg of commercial GAPDH was applied to each different steroid-BSA conjugate affinity columns and the elutes were analyzed by SDS-PAGE to identify the retained GAPDH by the different steroid-BSA conjugate affinity columns. **Lane 1**: E-6-BSA, **Lane 2**: P-3-BSA, **Lane 3**: T-3-BSA, **Lane 4**: C-21-BSA, **Lane 5**: BSA, **Lane 6**: Standard molecular weight marker. B. Competitive binding assay curve of commercial GAPDH using 17β-E-6-[^{125}I]-BSA as radiolabeled ligand, and 17β-E-6-BSA (triangle) and P-3-BSA (rectangle) as unlabeled ligands. Mean ± SE and the number of experiments are shown. "NO" indicates the absence of unlabeled ligands, and 17β-E-6-[^{125}I]-BSA represents ^{125}Iodine-labeled 17β-E-6-BSA. C. 17β-E-6-BSA affinity chromatography of commercial GAPDH (2.5 μg) in the presence and absence of free 17β-estradiol (1 μM). Retained GAPDH is shown in the absence of 17β-estradiol (**Lane 1**) which is competed off in the presence of 1 μM 17β-estradiol (**Lane 2**).

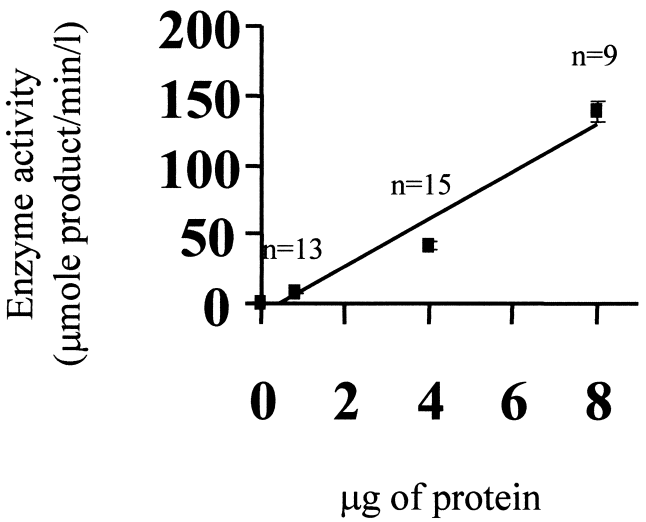

Fig. 6. Dose-dependent response of GAPDH. Enzymatic activity of GAPDH was measured at 340 nm wavelength for 60 s at 28–30°C. 0 μg, 0.8 μg (8 nM), 4 μg (40 nM), and 8 μg (80 nM) of GAPDH were tested in a total reaction volume of 670 μl at 15 mM G-3-P. The molar concentrations of GAPDH were calculated based on the molecular weight of the tetrameric GAPDH. Mean ± SE and the number of experiments are shown. In the 8 nM concentration, the SE overlaps within the size of the rectangle.

only the 17β-E-6-BSA conjugate binds to GAPDH, but that the non-protein-bound estrogen (17β-estradiol) also binds to GAPDH. When GAPDH (2.5 μg/1 ml, 17 nM) was applied to the 17β-E-6-BSA affinity column without incubation with 17β-estradiol, GAPDH was retained as shown in lane 1 (Fig. 5C, lane 1). In contrast, the GAPDH protein band was not detectable when the same amount of GAPDH was incubated with 17β-estradiol (final concentration was 1 μM) for 30 min at 4°C prior to the addition to the affinity column (Fig. 5C, lane 2), suggesting that 17β-estradiol impeded the binding of GAPDH to 17β-E-6-BSA completely.

Subsequently, we asked what would be the outcome of the interactions of free estrogen and progesterone with GAPDH. Since GAPDH is a classic glycolytic enzyme reducing NAD^+ to NADH in glycolysis, changes in enzymatic activity of GAPDH by the free steroids were expected. We employed an enzymatic assay in which reduction of NAD^+ to NADH by GAPDH was coupled with the catalysis of glyceraldehyde-3-phosphate (G-3-P) into 1,3-bisphosphoglycerate (1,3-BPG). The reduction of NAD^+ detected at 340 nm was measured by changes in the optical density at 28–30°C for 1 min. A dose-response relationship is depicted in Fig. 6. The concentrations of GAPDH (rabbit muscle) were 0 nM (0 μg), 8 nM (0.8 μg), 40 nM (4 μg) and 80 nM (8 μg) in a total reaction volume of 670 μl. The enzymatic activity for each dose of GAPDH was 0, 7.8 ± 0.8 (n = 13), 41.8 ± 3.3 (n = 15) and 138.2 ± 8.0 (n = 9) μmole of product/min/l, respectively. The activity of GAPDH was linearly related to the dosage of protein (r^2 = 0.96). Denatured GAPDH did not show enzymatic or binding activity (data not shown). Further, we tested the kinetics in the absence of glyceraldehyde-3-phosphate (G-3-P) or in the absence of NAD^+. In each case the optical density did not change for 1 min, confirming that the changes in optical density in the assay were solely due to the reduction of NAD^+ to NADH catalyzed by GAPDH (data not shown).

The effects of estrogen or progesterone on GAPDH kinetics were tested using the assay described above. The steroids or vehicle (ethanol) were added to the enzyme solution and the optical density at 340 nm wavelength was

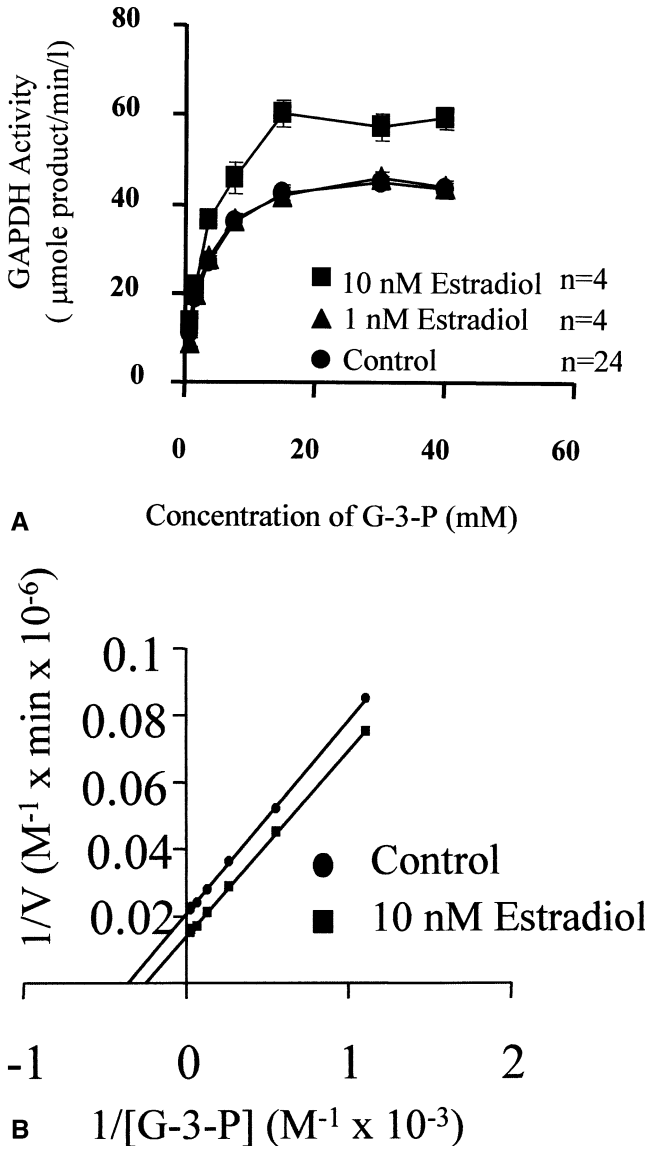

Fig. 7. The effect of different concentrations of 17β-estradiol on GAPDH activity. A. The activity of GAPDH (1 μg, 10 nM) is plotted as a function of different substrate (G-3-P) concentrations (0.9, 1.8, 3.75, 7.5, 15, 30 and 40 mM). The final concentrations of 17β-estradiol were 1 nM and 10 nM. The control samples received 1.4% ethanol. Mean ± SE and the number of experiments for control, 1 nM and 10 nM 17β-estradiol treatments are shown. B. Double-reciprocal plot (Lineweaver-Burk plot) of GAPDH activity for control and 10 nM 17β-estradiol treatment groups. It shows that both V_{max} and K_m of GAPDH were increased by the addition of 17β-estradiol.

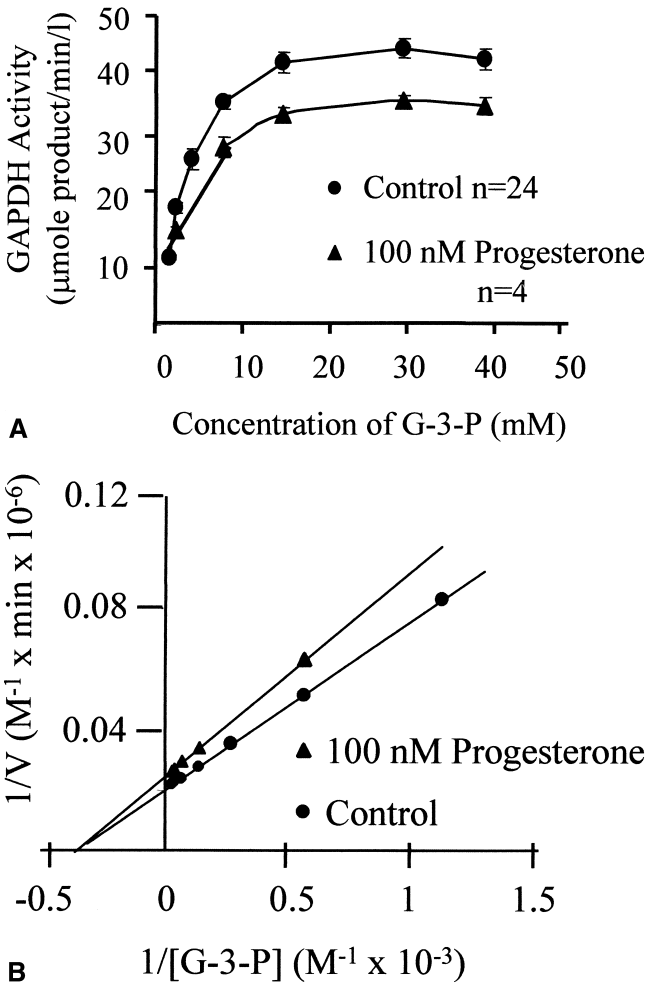

Fig. 8. The effect of different concentrations of progesterone on GAPDH activity. A. The activity of GAPDH (1 μg, 10 nM) was plotted as a function of different substrate (G-3-P) concentrations (1.8, 7.5, 15, 30 and 40 mM) in presence of 100 nM of progesterone. 1.4% (final concentration) ethanol was added to the control group. Mean ± SE and the number of experiments for control and 100 nM of progesterone-treated solutions are shown. B. Double-reciprocal plot (Lineweaver-Burk plot) of GAPDH activity for control and 100 nM progesterone treated groups. A decrease of V_{max} by the addition of 100 nM progesterone can be observed.

measured for 1 min at 28–30°C after brief mixing. In Fig. 7A the activity of GAPDH (1 μg, 10 nM) was plotted as a function of different G-3-P concentrations (0.9, 1.8, 3.75, 7.5, 15, 30 and 40 mM) in the presence of different concentrations of 17β-estradiol (1 and 10 nM) or ethanol (control group). In the control group the GAPDH activity began to plateau at 15 mM of G-3-P and it was maintained at 30 and 40 mM of G-3-P, showing saturation of the enzyme with its substrate. This was true for all the other experimental groups. 17β-Estradiol at a concentration of 1 nM did not alter the V_{max} and K_m. However, the V_{max} and K_m were increased significantly by the addition of 10 nM 17β-estradiol ($P < 0.01$). The double-reciprocal plot shows that the V_{max}'s of control and 10 nM 17β-estradiol-treated samples were 49.9 ± 2.1 and 68.6 ± 4.9 μmole product/min/l and that the K_m's were 3.1 ± 0.1 and 3.8 ± 0.4 mM, respectively (Fig. 7B). 10 nM 17β-Estradiol increased the V_{max} and K_m of GAPDH by about 37% and 23%, respectively. Other concentrations of 17β-estradiol (0.5 pM, 1 pM, and 0.5 nM) did not change the V_{max} or K_m of GAPDH (data not shown).

The effect of progesterone on the enzymatic activity of GAPDH was also tested (Fig. 8). Two concentrations of progesterone were tested, 10 nM and 100 nM (final concentration). The V_{max} of the controls was the same as that of the previous data (Fig. 7). The V_{max}'s of control, 10 nM and 100

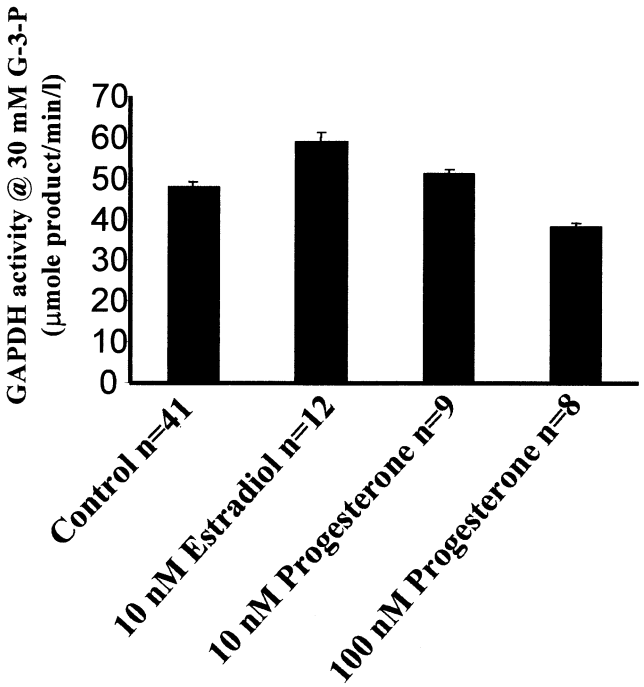

Fig. 9. Changes in GAPDH activity in the presence of 17β-estradiol or progesterone tested at a 30 mM G-3-P (substrate) concentration. n indicates number of experiments. Mean ± SE values are shown.

nM of progesterone-treated samples were estimated by double-reciprocal plot and were 49.9 ± 2.1, 56.6 ± 0.6, and 39.5 ± 1.4 μmole product/min/l, respectively. Also, the K_m's of control, 10 nM, 100 nM progesterone treated samples were 3.1 ± 0.1, 2.8 ± 0.1, and 2.8 ± 0.1 mM. The decrease in V_{max} down to 21% by 100 nM progesterone was significantly different from control ($P < 0.01$), whereas K_m was not changed (Fig. 8A, 8B). The apparent increase in V_{max} by 10 nM progesterone was not reliable because this increase was not replicated in another set of experiments (Fig. 9).

Fig. 9 shows that 10 nM 17β-estradiol increases the GAPDH activity by about 20%, and 100 nM progesterone decreased it by about 21% as expected. In contrast, 10 nM progesterone did not affect the enzymatic activity. Table 1 shows no significant changes in GAPDH activity by testosterone, corticosterone, 17α-estradiol, and diethylstilbestrol (DES). The small variation of GAPDH activity in each control condition was not significant ($P > 0.05$). Although 10 nM 17β-estradiol did not change the activity as much as our previous results, there was still a significant increase in GAPDH activity compared to the control ($P < 0.05$). Two concentrations of corticosterone, testosterone, 17α-estradiol and diethylstilbestrol did not show any significant effect on GAPDH activity ($P > 0.05$). Though there was a tendency to decrease the activity by the α-isomer and to increase it by DES. These results confirm not only the activating effect of 10 nM 17β-estradiol and the inhibiting effect of 100 nM progesterone on the enzymatic activity of GAPDH, but also the selective and specific interactions of GAPDH with estradiol and progesterone.

Table 1
Effects of 17β-estradiol, progesterone, corticosterone (corti), testosterone (testo), 17α-estradiol, and diethylstilbestrol (DES) on GAPDH activity using 30 mM G-3-P as substrate. N indicates number of experiments. Mean ± S.E. are shown. ANOVA was done to test differences between control and experimental groups for each condition.

Condition	N	GAPDH activity @ 30 mM G3P (μmole product/min/l)	*P* Value
Control	3	39.0 ± 0.5	
17β-Estradiol 10 nM	3	44.0 ± 1.5	<0.05
Control*	41	47.9 ± 1.4	
Progesterone 100 nM*	8	38.1 ± 1.0	<0.01
Control	3	37.0 ± 1.7	
Corti 10 nM	3	37.0 ± 2.4	N.S
Corti 100 nM	3	36.5 ± 1.7	N.S
Control	4	38.7 ± 2.3	
Testo 10 nM	4	37.7 ± 3.2	N.S
Testo 100 nM	4	35.7 ± 0.8	N.S
Control	8	37.0 ± 1.7	
17α-Estradiol 10 nM	8	34.2 ± 1.3	N.S
17α-Estradiol 100 nM	8	33.1 ± 1.4	N.S
Control	8	40.1 ± 1.5	
DES 10 nM	4	42.3 ± 3.4	N.S
DES 100 nM	4	43.6 ± 2.8	N.S

*GAPDH activities are taken from data in Fig. 9.

4. Discussion

The three major findings reported in this paper are: first, the isolation and purification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 37 kDa) from rat brains by both 17β-E-6-BSA and P-3-BSA affinity chromatography. The second finding is that 17β-estradiol has higher binding affinity to GAPDH than other steroids such as progesterone or testosterone. The third finding is that both 17β-estradiol and progesterone regulate the catalytic activity of GAPDH: 10 nM estradiol increases the V_{max} and K_m of GAPDH up to 37% and 23%, respectively, whereas 100 nM progesterone decreases only the V_{max} of GAPDH to 21%. Their effects are rather selective because 17α-estradiol, testosterone, DES, and corticosterone did not have any effects on its enzyme activity at either 10 nM or 100 nM concentrations.

Initially, the purification of GAPDH from unwashed plasma membrane-microsomal fractions from rat brains by progesterone affinity chromatography suggested an association of this cytosolic protein with the membrane fraction as reported by several laboratories [25–27]. However, a subsequent wash step of the final pellet containing the membrane fraction drastically reduced the presence of the enzyme, as shown by SDS-PAGE analysis and Western blot analysis. Furthermore, the enzyme activity of GAPDH in washed membrane fractions decreased dramatically compared to that of GAPDH in unwashed membrane fractions

(data not shown). It seems then logical to conclude that the purified GAPDH from P3 fractions was due to contamination with the cytosol of rat brains during subcellular preparation. However, although GAPDH has been known as a soluble cytosolic protein for a long time, there is much evidence showing that GAPDH can be considered also as a cell surface protein. For example, GAPDH is specifically bound to the inner surface of the human erythrocyte membrane [25] as well as to cardiac sarcolemmal and sarcoplasmic reticular membranes [26]. In prokaryotes, *Staphylococcus aureus* and *Staphylococcus epidermidis*, GAPDH has been identified as a cell surface-associated transferrin-binding protein in a cell wall [27]. Thus, it can not be ruled out completely that the GAPDH was associated with the plasma membrane of brain cells in a reversible and charged-dependent manner, so that it could be easily separated from the membrane by an additional stepwash procedure [26].

Because steroid-BSA conjugates were used in the affinity purification, it was necessary to assure that the purification of GAPDH was not due to non-specific interactions between BSA and GAPDH. SDS-PAGE analysis of GAPDH eluted from the BSA-affinity column did not show the 37 kDa protein band, indicating that BSA alone did not bind to GAPDH. Moreover, free estrogen (17β-estradiol) competed with the 17β-E-6-BSA conjugate, preventing the binding of GAPDH to the complex and indicating little or no interaction between BSA and GAPDH but a specific interaction of the steroid to the enzyme. In addition, different steroid hormones bound to GAPDH with different degrees of affinity. Estrogen bound to GAPDH with higher affinity than progesterone and testosterone, whereas corticosterone did not bind to GAPDH. The enzyme activity of GAPDH was changed by 17β-estradiol and progesterone, but not affected by testosterone, corticosterone, 17α-estradiol, or DES. According to their structural difference (see Fig. 4), we speculate that the presence of the β-orientated hydroxyl group at C-17 in the 17β-estradiol may be responsible for enhancing the binding and enzymatic activity. Changes in the orientation of the C-17 hydroxyl group or modifications, such as in progesterone, tend to decrease the activity. Future studies will be needed to investigate further these interactions.

It was of interest to find that 10 nM estradiol and 100 nM progesterone clearly altered the catalysis of GAPDH, but in opposite direction. The possible physiological relevance of these findings has been partially confirmed in our recent studies. We have investigated the enzyme activity of GAPDH in cytosolic and plasmalemma-microsomal (P3) fractions from the cerebellum and hippocampus of female rat brains during the rat estrous cycle. The GAPDH activity in both cytosolic and P3 fractions from the hippocampus and cerebellum were significantly reduced by 35–50% from proestrus to estrus, and they were about 59% higher in proestrus than in male rat brains (unpublished observation). These preliminary changes in in vivo catalysis are consistent with expected changes in level of estrogen (high in proestrus) and progesterone (high in estrus) during the rat estrous cycle.

GAPDH is a tetrameric enzyme, solubilized monomers or dimers are inactive and its catalytic activity depends on the active center of the tetrameric enzyme [28,29]. Thus, it is plausible that 17β-estradiol could facilitate changes in the tertiary structure of each monomer, resulting in the formation of a better fit for the substrate binding sites with subsequently increases in both the $V_{\max}$ and K_m of GAPDH. However, our data show that progesterone behaves like a non-competitive inhibitor obeying Michaelis-Menten kinetics. It seems that progesterone could bind to both the enzyme and enzyme-substrate complex causing inactivation of the catalysis of GAPDH.

Although mechanisms by which these steroid hormones modify the GAPDH activity are unclear, this unexpected finding demonstrates that estradiol and progesterone within physiological concentrations can regulate the activity of GAPDH in opposite directions and, therefore, control cell metabolism under specific physiological conditions, like in proestrus (high level of estrogen) or during pregnancy (high level of progesterone) in the rat.

This study of rapid and direct effects of estradiol and progesterone on GAPDH may have physiological importance. GAPDH is one of the proteins which plays role in bundling of microtubules [30]. This fact is supported by studies of the binding constant and stoichiometry of the GAPDH/microtubule complex [31,32]. It is suggested that glycolytic enzymes associated with the microtubule complex provide the energy for movement of MAPs and other proteins that bind to microtubules, and in the reorientation of intracellular organelles [33]. Therefore, estrogen and progesterone could act on glycolytic enzymes such as GAPDH to regulate the cellular energy required for these intracellular events and for the cross-link of microtubules with actin filaments responsible for changes in cell structure or coordination of cell motile processes. In this regard, it is then interesting to consider the role of non-genomic effects of estradiol and progesterone on GAPDH in the remodeling of synaptic morphology induced by estradiol, and after ovariectomy in rat brains [34–36]. It is speculated that the changes of GAPDH enzymatic activity by estradiol and progesterone could result in the modification of the cellular energy supply for the formations of microtubule bundles and the microtubule/actin filament complex, which is a critical step for alteration of synaptic morphology. Future studies will address these issues.

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