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Specific Binding of Dehydroepiandrosterone Sulfate to a Cytoplasmic Macromolecule in Human Fetal Membrane

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The cytoplasmic dehydroepiandrosterone 3-sulfate (DHAS)-binding macromolecule in the cytosol fraction of human fetal membrane has been investigated. It was distinguished from serum DHAS-binding component on the basis of binding specificity and molecular weight. Scatchard analysis indicated a single class of binding sites (57 fmol/mg protein) with an apparent dissociation constant of 7.2×10^{-8} M. The cytoplasmic DHAS-binding macromolecule was separated into two moieties by Sephacryl S-300 chromatography. One was eluted at the void volume, and the other hand a molecular weight of about 1.7×10^5. These two moieties had the same specificity for DHAS, and had no affinity for dehydroepiandrosterone or 17β-estradiol. Progesterone, 5α-dihydrotestosterone and sulfated estrogens such as 17β-estradiol 3-sulfate, estriol 3-sulfate and estrone 3-sulfate showed much lower affinity for the DHAS-binding macromolecule. Temperature-dependent nuclear uptake of [^{3}H]DHAS in a cell-free system required the presence of cytosol, suggesting that binding of DHAS to the cytoplasmic macromolecule is a prerequisite for the transfer of DHAS to the nucleus. These data demonstrate the presence in human fetal membrane of a tissue-specific DHAS-binding macromolecule which has properties similar to those of the DHAS-binding protein previously found in rabbit uterine cervix (A. Ito, K. Sakyo, H. Sano, S. Hirakawa and Y. Mori, *Chem. Pharm. Bull.*, **34**, 2118 (1986).

Keywords——dehydroepiandrosterone sulfate; dehydroepiandrosterone sulfate binding macromolecule; human amnion; human fetal membrane

Dehydroepiandrosterone 3-sulfate (DHAS) is an androgen produced by the adrenal gland.[1] Recently, we have reported that the administration of DHAS to rabbits in the middle stage of gestation accelerates the cervical dilatation *in vivo*.[2] In cervical tissue and cell cultures, addition of DHAS to the culture media promotes collagenase production, while that of 17β-estradiol (E_2) or dehydroepiandrosterone (DHA) does not.[3,4] In another report, we have demonstrated the presence of DHAS-binding protein in the cytosol fraction of rabbit uterine cervix and have shown that its characteristics are similar to those of other well-known steroid hormone receptors.[5] These studies suggest that DHAS-binding protein is able to act as an effective concentrating agent or a hormone receptor.

The present study, therefore, was designed to identify another target tissue of DHAS, in order to provide a basis for further studies on the mechanisms of DHAS action. Fetal membrane seems the most probable site of prostaglandin synthesis to generate myometrial contractions and the onset of labor in women,[6] and DHAS has been reported to activate phospholipase A_2 in human fetal membrane,[7] suggesting that fetal membrane may also be a target tissue of DHAS. In this paper, we present evidence for the presence of a specific DHAS-binding macromolecule in human fetal membrane, and describe some of its properties.

Materials and Methods

Materials——The following reagents were commercially obtained: [7-^{3}H(N)]dehydroepiandrosterone 3-sulfate ([^{3}H]DHAS) ammonium salt (35 Ci/mmol), which was checked for purity (more than 99%) by thin layer chromatography within 2 months before use, from New England Nuclear, Boston, Mass., U.S.A.; α-chymotrypsinogen A from Sigma, St. Louis, Mo., U.S.A.; egg albumin (5×, crystallized) from ICN Pharmaceuticals, Cleveland, Oh., U.S.A.; Sephadex G-100 from Pharmacia, Uppsala, Sweden. All other reagents used were described in a previous report.[5]

Preparation of Cytosol of Human Fetal Membrane and Binding Assays——The following experimental procedures were all performed at 4 °C unless otherwise stated.

Fetal membrane free from placenta was immediately excised after spontaneous labor from women at 38—40 weeks of gestation. If necessary, the amnion was manually separated from the remainder of the tissue. These tissues were rinsed with cold saline to remove clotted blood, and stored at −80 °C until use. The stored tissues were used within two months.

The minced fetal membrane (usually about 1 g) was homogenized in 4 vol. of ice-cold 20 mM Tris–HCl/3 mM EDTA/12 mM thioglycerol/10 mM Na_2MoO_4/20% (v/v) glycerol, pH 7.4 (TETgMoG-20% buffer) in a glass homogenizer. To prepare cytosol fraction, the homogenate was centrifuged at 105000×*g* for 60 min at 4 °C in a Hitachi 55P ultracentrifuge using a type RP65T rotor.

The cytosol (2.0—3.7 mg protein/ml) was incubated for 16 h at 4 °C with 136 nM [^{3}H]DHAS in the presence or absence of 27.2 μM unlabeled DHAS. To examine the binding activity of serum component, the serum from the above pregnant women was diluted 1:24 (v/v, 3.5 mg protein/ml) with TETgMoG-20% buffer and incubated with [^{3}H]DHAS as described above.

Gel-Exclusion Chromatography——Sephadex G-50 chromatography was performed to remove unbound [^{3}H]DHAS as described elsehwere,[5] using a small Sephadex G-50 column (1.2×14 cm) previously equilibrated with 20 mM Tris–HCl/3 mM EDTA/12 mM thioglycerol/10 mM Na_2MoO_4/10% (v/v) glycerol, pH 7.4 (TETgMoG-10% buffer). Fractions of 1 ml were collected and aliquots of 0.5 ml from each fraction were counted for radioactivity in 8 ml of ACS II scintillation fluid (Amersham Corp., Bucks., U.K.) using an Aloka 903 liquid scintillation counter.

To distinguish the cytoplasmic DHAS-binding macromolecule from serum DHAS-binding component, a calibrated Sephadex G-100 column (1.2×60 cm) equilibrated previously with TETgMoG-10% buffer was used. The same buffer was used for elution (flow rate 15 ml/h), and aliquots of 0.5 ml from each fraction (1 ml) were counted as above. The column was calibrated with blue dextran, bovine serum albumin (BSA) (6.7×10^4), egg albumin (4.5×10^4), and α-chymotrypsinogen A (2.57×10^4).

For further analysis of the molecular weight of cytoplasmic DHAS-binding macromolecule, Sephacryl S-300 chromatography was carried out as described previously.[5]

Transfer of DHAS-Binding Macromolecule Complex from Cytosol to Nucleus——In this experiment, preparation of cytosol, incubation of cytosol with [^{3}H]DHAS and Sephadex G-50 chromatography were carried out using molybdate-free buffers. Removal of molybdate from the buffers did not change the amount of specific binding (data not shown). The other procedures and conditions including preparation and purification of the nuclei from fetal membrane were the same as previously described.[5]

All other experimental procedures were described in a previous report.[5]

Results

Specific Binding of [^{3}H]DHAS to Cytosol Components in Human Fetal Membrane

The cytosol fraction prepared from fetal membrane was incubated with various concentrations of [^{3}H]DHAS in the presence or absence of a 200-fold excess of unlabeled DHAS, and the specific binding was estimated. As shown in Fig. 1A, [^{3}H]DHAS specific binding increased with increasing concentration of [^{3}H]DHAS in the incubation mixture, and the specific binding was saturable at about 100 nM added [^{3}H]DHAS. From the Scatchard plots[8] of these data (Fig. 1B), the K_d and the number of binding sites were calculated to be 7.2×10^{-8} M and 57 fmol/mg protein, respectively. These kinetic constants are similar to those for the DHAS-binding protein in rabbit uterine cervix.[5] The specific binding at 136 nM, which was usually 40—60% of total binding, was not altered by exposure to a 200-, 500-, or 1000-fold excess of unlabeled DHAS (data not shown). An identical result was obtained when the cytosol from amnion alone (separated from fetal membrane) was examined. The following experiments, therefore, were carried out using whole fetal membrane.

The effect of incubation time on specific binding at 4 °C was also examined. The maximal

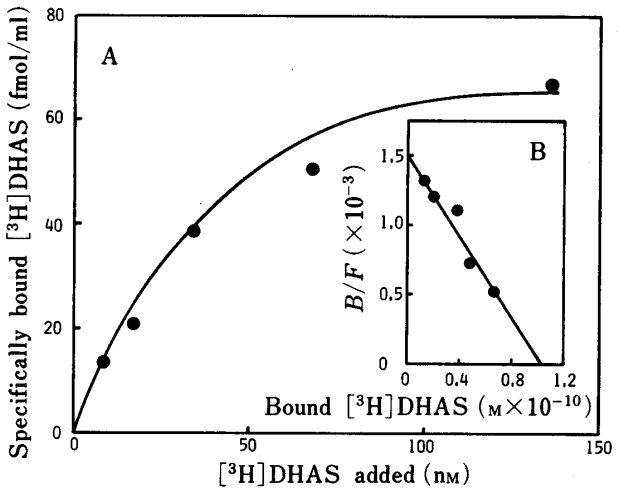

Fig. 1. Specific Binding of [^{3}H]DHAS to Cytoplasmic Macromolecule of Human Fetal Membrane

A. Specific binding of [^{3}H]DHAS as a function of the concentration of [^{3}H]DHAS. The cytosol prepared from human fetal membrane at 40 weeks of gestation was incubated for 16 h at 4 °C with various amounts of [^{3}H]DHAS as indicated in the presence or absence of a 200-fold excess of unlabeled DHAS, and then aliquots (0.5 ml) of the labeled cytosol were applied to a Sephadex G-50 column to remove unbound steroid. Specific binding was calculated from the difference in binding of [^{3}H]DHAS in the presence and absence of unlabeled DHAS. Other experimental details are described in the text.

B. Scatchard analysis for the cytoplasmic DHAS-binding macromolecules in human fetal membrane. *B/F*, bound-to-free ratio. Data are representative of two identical experiments.

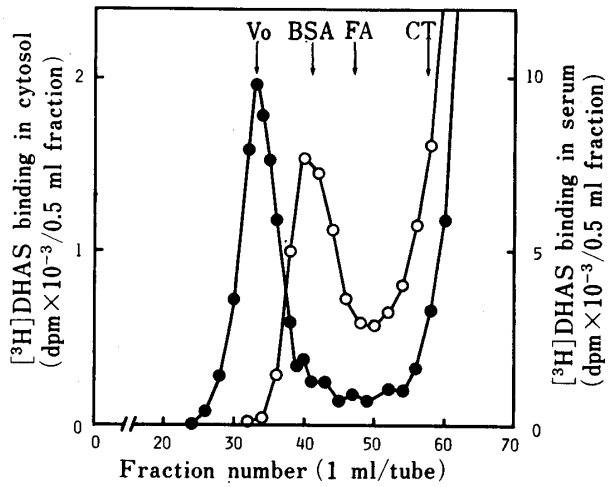

Fig. 2. Sephadex G-100 Chromatography of the [^{3}H]DHAS-Binding Components in the Cytosol from Fetal Membrane and in Serum

The cytosol fraction (1 ml, ●) prepared from human fetal membrane or diluted serum (1 ml, ○) from the same woman was incubated for 16 h at 4 °C with 136 nM [^{3}H]DHAS, and then applied to a Sephadex G-100 column. Other experimental conditions are described in the text. The arrows indicate the elution positions of blue dextran (Vo), BSA, egg albumin (EA), and α-chymotrypsinogen A (CT).

binding was observed at 4 h of incubation and was maintained for at least 16 h (data not shown).

Formation of labeled metabolites does not appear to be a complicating factor in the present study, since only 3.5% of the radioactivity, which may be that of DHA, 5α-dihydrotestosterone (DHT) or testosterone (T) as judged by thin layer chromatography, was found to represent the metabolites of [^{3}H]DHAS after incubation for 16 h at 4 °C (data not shown).

The nature of binding of [^{3}H]DHAS in serum prepared from a woman at the same stage of gestation was different from that in the fetal membrane in that there was no competition in the presence of excess unlabeled DHAS (data not shown). These results suggest the presence of a specific DHAS-binding macromolecule in the cytosol of human fetal membrane. This conclusion was further confirmed by the elution patterns from Sephadex G-100 (Fig. 2). The molecular weight of the DHAS-binding component in serum was almost the same as that of BSA, while the cytoplasmic DHAS-binding macromolecule was excluded from the gel column. Furthermore, if only the contaminating serum protein were responsible for the small amount of [^{3}H]DHAS binding seen in the cytosol (fraction nos. 39—48), the extent of contamination would have to be up to 10%. Therefore, the specific binding of DHAS in fetal membrane is due to the specific macromolecule present in this tissue.

Characterization of Cytoplasmic DHAS-Binding Macromolecule in Human Fetal Membrane

As shown in Fig. 3, the DHAS-binding macromolecule was separated into two moieties on Sephacryl S-300 chromatography. One was eluted at the void volume, and the other had a

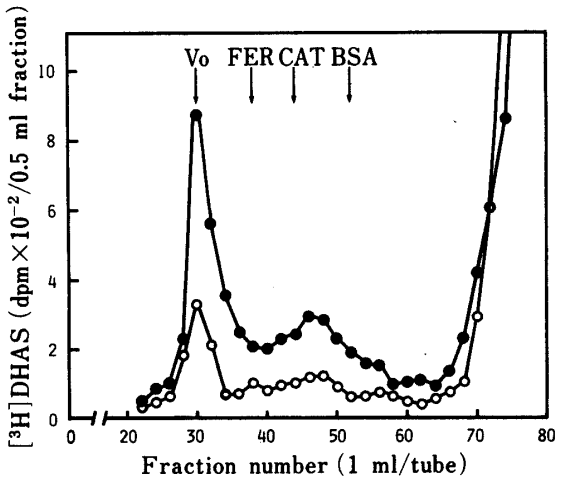

Fig. 3. Elution Pattern of the Cytoplasmic DHAS-Binding Macromolecules in Human Fetal Membrane from a Sephacryl S-300 Column

The cytosol fraction (1 ml) prepared from human fetal membrane was incubated for 16 h at 4 °C with 136 nM [^{3}H]DHAS in the presence (○) or absence (●) of a 200-fold excess of unlabeled DHAS, and applied to a Sephacryl S-300 column. Other experimental details are described in the text. The arrows indicate the elution position of blue dextran (Vo), ferritin (FER), catalase (CAT), and BSA. Data are representative of five identical experiments.

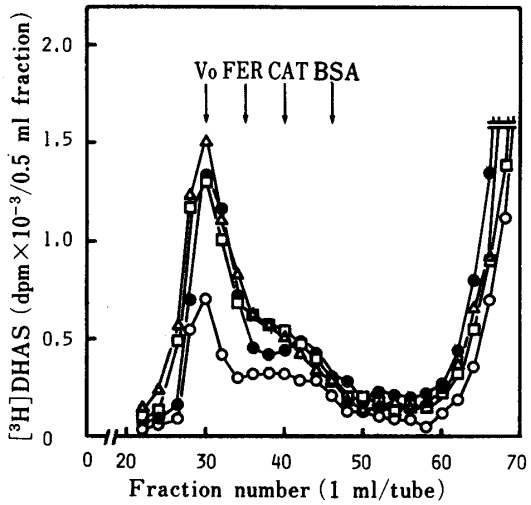

Fig. 4. Effect of DHA and E_2 on the Binding of [^{3}H]DHAS to Cytoplasmic DHAS-Binding Macromolecules

The cytosol fraction (1 ml) from human fetal membrane was incubated for 16 h at 4 °C with 136 nM [^{3}H]DHAS in the absence (●) or presence of a 200-fold excess of unlabeled DHAS (○), DHA (△), or E_2 (□), and applied to a Sephacryl S-300 column. Other experimental conditions are described in the text. Arrows are the same as in Fig. 3. Data are representative of three identical experiments.

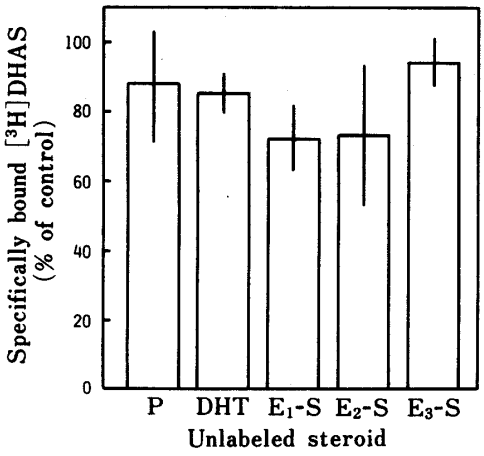

Fig. 5. Effect of Unlabeled Steroids on the Binding of [^{3}H]DHAS to Cytoplasmic Macromolecules in Human Fetal Membrane

The cytosol from human fetal membrane was incubated for 16 h at 4 °C with [^{3}H]DHAS (136 nM) in the presence or absence of a 200-fold excess of unlabeled steroids as indicated. Bound radioactivity was determined by using gel filtration on Sephadex G-50. Other experimental conditions were the same as those in Fig. 1. The specific binding of [^{3}H]DHAS corresponded to 95.9 ± 9.7 fmol/ml cytosol (7451 ± 753 dpm), which is taken as 100% binding. Data are presented as the mean ± S.D. of three separate experiments. E_1-S, estrone 3-sulfate; E_2-S, 17β-estradiol 3-sulfate; E_3-S, estriol 3-sulfate.

molecular weight of about 1.7×10^5. Both moieties had about the same specific binding capacity, though the larger molecule showed a relatively sharp elution peak. Recently, molybdate has been used extensively to stabilize the large molecular forms (8—10 S) of other well-known classical steroid hormone receptors.[9-12] However, no detectable difference was seen in the Sephacryl S-300 elution patterns of the DHAS-binding macromolecules in human fetal membrane in the presence of and in the absence of molybdate (data not shown).

Figure 4 shows the effects of DHA and E_2 on the specific binding of DHAS to the cytoplasmic macromolecules. DHA and E_2 showed no affinity for either of the two DHAS binding peaks. Thus, the two DHAS-binding components apparently show the same properties as regards affinity for various steroids. The effects of some other steroids were investigated by using a Sephadex G-50 column. As shown in Fig. 5, although E_2 3-sulfate and

TABLE I. Effect of Cytosol on Uptake of [^{3}H]DHAS by Purified Nuclei of Human Fetal Membrane

Incubation of purified nuclei in the presence of:	Translocation of [^{3}H]DHAS in the nuclei (dpm/μg DNA)	
	0 °C	25 °C
[^{3}H]DHAS bound to cytosol	19.5 ± 2.4 (3.1)	47.0 ± 3.3[a] (7.5)
[^{3}H]DHAS and BSA	1.9 ± 1.1 (0.2)	4.0 ± 3.5 (0.4)

The purified nuclei (18.3 μg DNA) from human fetal membrane were incubated at 0 or 25 °C for 6 h with the prelabeled cytosol (1.2×10^4 dpm; 154 fmol) or a buffer containing [^{3}H]DHAS and BSA (1.7×10^4 dpm; 219 fmol). Nuclei were reisolated by centrifugation at $800 \times g$ for 10 min at 4 °C, washed, and counted for radioactivity. Other experimental conditions are described in the text. Each value represents the mean ± S.D. of four determinations. *a*) Significantly different from the value at 0 °C ($p < 0.001$). The percentage of total radioactivity which was taken up by the nuclei is given in parenthesis.

estrone 3-sulfate were able to compete with [^{3}H]DHAS for the binding site to the extent of about 30%, DHT, progesterone, and estriol 3-sulfate showed lower affinity for the DHAS-binding macromolecules. These results apparently indicate that the binding of DHAS to the macromolecules is highly specific, and that the sulfated region of DHAS is essential to the binding, which is not due to a nonspecific interaction between a charged sulfated region and the macromolecules.

Retention of [^{3}H]DHAS by the Nuclei in Cell-Free Systems

We investigated whether binding of [^{3}H]DHAS to the cytoplasmic macromolecule is a prerequisite for the transfer of the hormone to the nucleus, since such a two-step mechanism is well known to operate in other steroid receptor systems. Table I shows the effect of cytosol on uptake of [^{3}H]DHAS by purified nuclei of fetal membrane. In the presence of cytosol, [^{3}H]DHAS could be taken up effectively by nuclei, and the amount of radioactivity incorporated into nuclei at 25 °C was about 2.5-fold more than that at 0 °C. In the absence of cytosol, almost no radioactivity was taken up by nuclei. These results suggest that [^{3}H]DHAS first combines with cytoplasmic macromolecules and then the complex formed interacts with the nucleus in a temperature-dependent process to form the nuclear complex.

Discussion

It is well known that DHAS is changed into androgens by the action of sulfatase and Δ^4-3β-hydroxysteroid dehydrogenase (3β-HSD) and then into E_2 by aromatase, and monkey placenta contains much 3β-HSD and aromatase.[13] Recently, human fetal membrane also has been reported to contain sulfatase activity.[14] However, only minute amounts of metabolites of [^{3}H]DHAS resulting from the action of such enzymes were found to contribute to the binding in cytosol, as described above. Furthermore, sulfates of estrogens could not compete with [^{3}H]DHAS effectively for the specific binding sites (see Fig. 5). Thus, the metabolites of [^{3}H]DHAS have little involvement in the binding activity under the conditions used.

Although we have already reported the presence of cytoplasmic DHAS-binding protein in rabbit uterine cervix,[5] the present report is the first concerning a specific DHAS-binding macromolecule in human tissues. The DHAS-binding macromolecule in fetal membrane was eluted from Sephacryl S-300 in the fractions corresponding to the molecular weight of 1.7×10^5 and in the void volume fractions in a ratio of about 1 : 1. The DHAS-binding protein in rabbit cervix was also eluted in the same fractions, but in this case, molecular weight 1.7×10^5 moiety was predominant. Specific binding was not observed clearly when the

isolated cytosol was used for the radio-labeling in the case of rabbit cervix, whereas specific binding occurred in the isolated cytosol of human fetal membrane. Furthermore, as well as the case of rabbit cervix, we recently have observed that the cytosol fraction of human uterine cervix contains specific DHAS-binding component that is eluted from Sephacryl S-300 mainly in the fractions corresponding to the molecular weight of 1.7×10^5, and also in the void volume in a small amount; such a clear specific binding was not seen when the binding of DHAS was performed after the cytosol was isolated (K. Sakyo, A. Ito, S. Hirakawa and Y. Mori, unpublished data). These findings suggest that the unoccupied DHAS-binding protein of uterine cervix is very labile before the ligand is bound to the protein, and may be destroyed by mechanical homogenization and/or by some neutral proteases released during the homogenization, whereas the cytosol of fetal membrane may be relatively free of such enzymes so that the DHAS-binding macromolecule is stable or tends to aggregate. Some heterogeneity between the DHAS-binding protein of rabbit cervix and that of human fetal membrane was also seen in the steroid specificities; *i.e.*, DHA showed some affinity for the specific DHAS-binding site of the cytosol of rabbit uterine cervix, while DHA showed no affinity for that of fetal membrane. In both cases, however, we confirmed that only small amounts of metabolites of [^{3}H]DHAS were produced during incubation under the conditions used, and the binding of DHAS to the cytoplasmic components was not due to nonspecific binding between the charged sulfated region of DHAS and the components. Both DHAS-binding components have almost the same high K_d values (10^{-8} M order) and a limited number of binding sites (about 10^{-14} mol/mg protein), although the Scatchard analysis in Fig. 1 may be incomplete for the reason discussed in a previous report.[5] It is interesting that both DHAS-binding components were able to translocate to the nuclei in temperature-dependent processes. Thus, in spite of the difference in their origins, the two DHAS-binding components show considerable similarities in their properties. In addition, the properties described above are reminiscent of other well-known classical steroid hormone receptors, though it is still not clear whether the function and precise physicochemical characteristics of the DHAS-binding components are the same as those of the steroid hormone receptors. Further information on the acion of DHAS on fetal membrane is needed in order to understand the physiological role of the DHAS-binding macromolecule; so far, there has been only one report that DHAS stimulates the activity of phospholipase A_2 in human fetal membrane.[7]

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