Dehydroepiandrosterone sulfate-binding sites in plasma membrane from human uterine cervical fibroblasts

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Abstract. Dehydroepiandrosterone sulfate (DHA-S) plays a critical role in cervical dilatation at labor. Incubation of cervical fibroblasts with [^{3}H]DHA-S caused a rapid and saturable increase in cellular radioactivity: an apparent equilibrium was reached by 2 min. There was no detectable conversion of DHA-S into DHA or oestradiol. When the fibroblasts loaded with [^{3}H]DHA-S were homogenized and fractionated, the specific radioactivity in the plasma membrane fraction was enriched approximately 8- to 9-fold compared with the whole homogenate; only low amounts of radioactivity were observed in the other subcellular fractions. The binding of DHA-S to plasma membrane preparations showed saturation kinetics with an apparent equilibrium dissociation constant (K_d) of 12 nM, and the binding capacity (B_{max}) was calculated to be 1.25 fmol/mg protein. Neither DHA nor oestrone sulfate affected [^{3}H]DHA-S binding to the plasma membrane. The plasma membranes of skin fibroblasts did not show specific binding sites for DHA-S. These findings demonstrate the presence of specific binding sites for DHA-S in the plasma membrane of cervical stroma cells. The fetal adrenal steroid may exert its action on cervical ripening at least in part through membrane-associated binding sites, or receptors.
Key words. Human; uterine cervix; fibroblasts; dehydroepiandrosterone sulfate; ripening; labor.

During primate pregnancy the fetal adrenal gland produces the androgens dehydroepiandrosterone (DHA) and dehydroepiandrosterone sulfate (DHA-S), which are converted to oestrogens in the placenta [1, 2]. In particular, DHA-S has recently been shown to increase in parallel with the progress of pregnancy, with an accelerated rise shortly parturition [3, 4]. DHA-S is also known to promote cervical ripening directly or indirectly [5, 6]. The adrenal steroid may therefore be associated with cervical dilatation in late pregnancy and the induction of labor.

The uterine cervix consists mainly of fibrous connective tissue, such as collagen, which undergoes substantial remodeling during pregnancy. Explanations of the action of DHA-S on dilatation have focused on the increase in collagenolytic activity and in proteoglycan concentration in cervical matrix [7–10], although it remains unclear whether it is DHA-S or oestrogens derived from DHA-S in the placenta which plays the principal role in mediating cervical softening. It is generally assumed that, in responsive cells, steroid hormones diffuse passively through biomembranes to the nuclear receptors which determine the cellular specificity of response [11], such as cervical softening. But DHA-S is more readily accumulated and retained in uterine cervix than in tissues that are not its target [7–10]. It may be that it exerts its effects through specific binding sites (or receptors) in the cellular membrane, since some experiments indicate that DHA-S might interact with components of the cell membrane and may enter its target cells by a membrane-mediated process. These data prompted us to investigate DHA-S binding components associated with the plasma membranes from human cervical fibroblasts.

Materials and methods

Preparation of human cervical and skin fibroblasts. Small specimens of cervical endometrium were obtained from patients undergoing elective caesarean section, and small skin specimens were taken from the anterior abdominal wall at hysterectomy. The tissues were trimmed and minced under a laminar flow hood in HANKS' solution, and digested for 1 h at 37 °C with 0.25% collagenase (type I) dissolved in HANKS' solution [12]. Stroma and cell debris were removed by filtration through a nylon mesh. The filtrate was then centrifuged at 500 × g for 5 min. The resulting pellet was resuspended and incubated in RPMI 1640 medium supplemented with 12.5% fetal bovine serum. After several days of incubation, non-adherent cells were removed and the fibroblasts left to grow as a monolayer. The cells were harvested in 0.1% trypsin/0.02% EDTA and finally resuspended in medium or balanced salt solution (BSS, 135 mM NaCl, 4.5 mM KCl, 0.5 mM $MgCl_2$, 1.5 mM $CaCl_2$, 5.6 mM glucose, 10 mM HEPES, pH 7.4).

[^{3}H]DHA-S incorporation into fibroblasts. The fibroblasts, suspended at a concentration of 1×10^6 cells/ml in medium supplemented with 12.5% foetal bovine serum, were incubated with various concentrations of [^{3}H]DHA-S at 4 or 22 °C. In additional tubes, a 200-fold excess of DHA-S was added at the beginning of the incubation to evaluate non-specific uptake. At various time intervals the cell suspensions were diluted with 10 vol. of ice-cold BSS. The cells were then washed twice with ice-cold BSS to remove unincorporated [^{3}H]DHA-S, and were pelleted by centrifugation at 400 × g for 5 min. Radioactivity in the pellet was counted. When the reaction was terminated by adding 4 vol. of chloroform/methanol (1:2, v/v), followed by 1 vol. of chloroform and 1 vol. of H_2O, over 98% of the radioactivity was found in the water-soluble fraction (upper phase) rather than in the chloroform-soluble fraction (lower phase).

Subcellular distribution of incorporated [^{3}H]DHA-S. 50×10^6 cells were incubated with 1.5 nM [^{3}H]DHA-S

(1×10^5 dpm/nmol) at 4 °C for 16 h, or at 22 °C for 1 h. The cells were then harvested, washed and collected as described above. Ice-cold lysis buffer (1 mM EGTA, 10 mM Tris, pH 7.4) was added (4–5 times the cell pellet volume), and the cells were suspended and simultaneously lysed by rapid mixing on a vortex mixer for 30 s followed by brief sonication for 5 s. The homogenate was centrifuged at 800 × g for 10 min to remove nuclei and cell debris. The supernatant was centrifuged again at 100,000 × g for 1 h. The resulting supernatant was stored as the cytosolic fraction; the pellet was resuspended in a small amount of lysis buffer, layered on top of 35% (w/v) sucrose in lysis buffer and centrifuged at 100,000 × g for 1 h. The narrow band found at the interface was collected as the plasma membrane fraction, while the pellet was collected as debris from other organelles. Both these fractions were diluted with lysis buffer and centrifuged at 100,000 × g for 1 h. The final pellet was resuspended in lysis buffer and assayed immediately for radioactivity, protein concentration, and marker enzyme. The activity of 5′-nucleotidase was used as a marker for plasma membranes[13]. Protein was determined according to the method of Lowry et al.[14] using bovine serum albumin (BSA) as a standard.

[^{3}H]DHA-S binding to plasma membrane. DHA-S binding sites were examined in the plasma membrane preparations obtained from cervical or skin fibroblasts as described above. The values of K_d and B_{max} for [^{3}H]DHA-S-binding to plasma membrane fractions were determined according to the double reciprocal plots. Aliquots (0.1 ml) containing approximately 50 µg plasma membrane protein were incubated in triplicate with [^{3}H]DHA-S in the concentration range of $0.1 \sim 10 \times 10^{-9}$ M. A 200-fold excess of unlabelled DHA-S was added to paired samples for determination of displaceable or non-specific binding. In some experiments, the incubations were performed in the presence of DHA or oestrone sulfate. Incubations were carried out at 4 °C for 16 h. The membranes were separated by filtration, washed and counted. Specifically bound [^{3}H]DHA-S was calculated by subtracting binding in the presence of an excess of nonradioactive DHA-S from binding in its absence.

Statistics. Statistical analysis was performed by t-test. Differences were considered significant at $p < 0.01$.

Materials. [7-^{3}H(N)]DHA-S (23 Ci/mmol) was obtained from New England Nuclear. DHA-S and DHA were kindly donated from Kanebo, Japan. Oestrone sulfate was purchased from Sigma. All other chemicals were of reagent grade.

Results

DHA-S incorporation into whole cells. Figure 1 shows the time-course of DHA-S uptake by intact cervical fibroblasts from pregnant uterus and by skin fibroblasts. The specific accumulation shown here is the amount of radioactivity not displaced by the addition of a 200-fold excess of unlabeled DHA-S. When human cervical fibroblasts were incubated with 1.5 mM [^{3}H]DHA-S, the cellular radioactivity increased rapidly for 1 min and then reached a plateau. The initial rate of uptake was greater at 22 °C than at 4 °C. At both temperatures, accumulation of DHA-S was essentially maximal within 2 min, and there was no further increase in uptake of DHA-S during 90 min of incubation. Conversely, no significant retention was seen with the skin fibroblasts, which are not considered to be a target tissue for DHA-S.

When lipid (chloroform)-soluble and water-soluble fractions were isolated from fibroblasts incubated with [^{3}H]DHA-S, ^{3}H-radioactivity was found in the water-

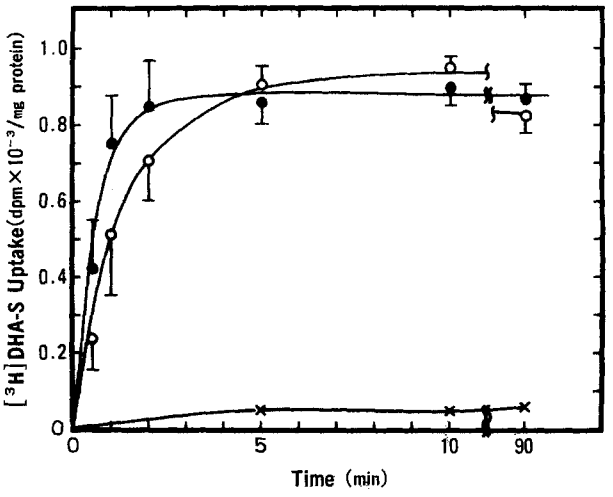

Figure 1. Time-course of specific [^{3}H]DHA-S incorporation into human cervical fibroblasts and skin fibroblasts. The cervical fibroblasts (2×10^6 cells) were incubated with 1.5 nM [^{3}H]DHA-S at 4 °C (o—o) or 22 °C (•—•). The skin fibroblasts (1×10^6 cells) (×—×) were incubated at 22 °C. Specifically incorporated DHA-S was calculated by subtracting uptake in the presence of excess nonradioactive DHA-S from uptake in the absence of excess unlabeled DHA-S. Each point is the mean ± SD of triplicate determinations. These data are representative of two similar experiments. * $p < 0.001$ cervical fibroblasts versus skin fibroblasts.

Subcellular distribution of incorporated [^{3}H]DHA-S into cervical fibroblasts and 5′-nucleotidase activity. The cervical fibroblasts (5×10^7 cells) were incubated with 1.5 nM [^{3}H]DHA-S at 4 °C for 16 h, or at 22 °C for 1 h. The subcellular fractions prepared as described in the text were subjected to radioactivity count and measurement of 5′-nucleotidase activity. Values are mean (SD) of two separate experiments performed in triplicate determinations. The number in [ ] gives the enrichment of the specific activity in a given membrane fraction relative to the specific activity of the homogenate

Fraction	^{3}H-Radioactivity dpm × 10^{-3}/mg protein			5′-Nucleotidase µM/mg protein/h		
4 °C						
Homogenate	2.58	(0.93)	[1.00]	1.33	(0.25)	[1.00]
Cytosol	1.98	(0.86)	[0.69]	0.35	(0.12)	[0.26]
Plasma membrane	22.96	(5.15)	[8.90]	8.12	(1.22)	[6.10]
Organellar debris	5.83	(0.97)	[2.26]	3.62	(0.63)	[2.72]
22 °C						
Homogenate	3.28	(2.04)	[1.00]	1.86	(0.56)	[1.00]
Cytosol	2.01	(2.53)	[0.61]	0.89	(0.45)	[0.48]
Plasma membrane	32.82	(5.64)	[10.01]	13.02	(2.45)	[7.00]
Organellar debris	4.37	(1.85)	[1.33]	5.58	(0.78)	[3.00]

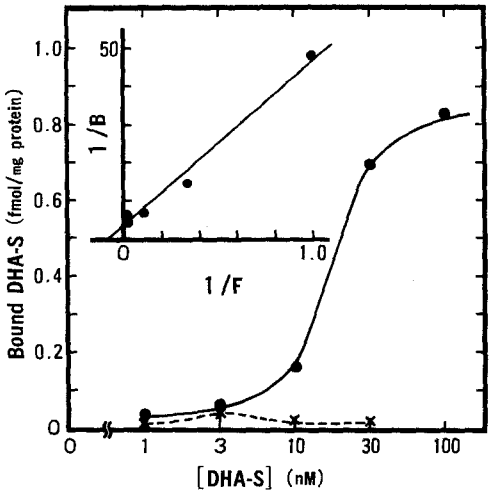

Figure 2. Specific binding of [^{3}H]DHA-S to plasma membranes isolated from cervical and skin fibroblasts as a function of DHA-S concentration. Plasma membrane preparations (50 μg protein) from cervical fibroblasts (●—●) or skin fibroblasts (×—×) were incubated with various concentrations of [^{3}H]DHA-S at 4 °C for 16 h. These data are representative of two similar experiments. The *inset* shows a double reciprocal plot of the data from which an equilibrium dissociation constant (K_d) of 12 nM and binding capacity (B_{max}) of 1.25 fmol/mg protein were calculated.

soluble but not in the lipid-soluble fraction, suggesting that little conversion of DHA-S to DHA or oestrogens occurs in cervical fibroblasts under these conditions.

Subcellular distribution of incorporated DHA-S. Intracellular binding sites for [^{3}H]DHA-S were examined in the cervical fibroblasts. The cells were incubated with [^{3}H]DHA-S at 4 °C or 22 °C and fractionated into cytosol, plasma membrane, and other organellar debris. As summarized in the table, the 5′-nucleotidase activity in the plasma membrane fraction was more than 6 times greater than that of the whole homogenate. Although contamination by other marker enzymes within the plasma membrane fraction was not measured, this fraction clearly consists of highly concentrated plasma membranes. The specific activity of ^{3}H-count was enriched approximately 8- to 9-fold compared with the whole homogenate prepared from cells incubated either at 4 °C or at 22 °C. No significant ^{3}H-radioactivity was detected in the nuclear fraction (data not shown). These findings indicate that the primary and significant binding of DHA-S occurs in the plasma membrane.

DHA-S binding to plasma membranes. To probe for specific DHA-S-binding sites in plasma membrane, the isolated membranes of cervical fibroblasts were incubated with various concentrations of DHA-S (fig. 2). The binding of [^{3}H]DHA-S showed saturation kinetics with an apparent K_d value of 12 nM, and B_{max} was calculated to be 1.25 pmol/mg protein. Neither DHA nor oestrone sulfate modified the DHA-S binding kinetics. As expected, skin fibroblast plasma membranes showed no evidence of specific binding sites for DHA-S.

Discussion

It is well known that repeated administration of DHA-S to pregnant women accelerates cervical ripening, shown as increase in wet weight of the cervix and in enzymes such as collagenase and alkaline proteinase in cervical tissue[5–10]. The actions of DHA-S have been attributed to oestrogens derived from DHA-S[7–10]. DHA-S is converted into DHA and testosterone by the action of sulfatase and Δ^4-3-β-hydroxysteroid dehydrogenase, and then into 17-β-oestradiol by the action of aromatase in the placenta[1,2]. As an alternative pathway, DHA-S is also known to be 16-α-hydroxylated in the liver, then aromatized to oestriol in the placenta[1,2]. However, oestrogens and DHA are reported to inhibit collagenolytic activity and suppress production of the enzymes involved in cervical ripening[15–17]. Consequently it is likely that unchanged DHA-S rather than its metabolites, mediates the effects of DHA-S on cervical ripening. Since specific DHA-S accumulation could be demonstrated in cervical fibroblasts and since little conversion of DHA-S to DHA or oestrogens was observed, DHA-S may act directly on the cervical stromal cells.

Prostaglandins may also stimulate cervical ripening, and an increase in prostaglandin synthesis within the uterus appears to be a common feature in labor[18,19]. DHA-S induces mobilization of the precursor arachidonic acid from membrane phospholipids, and consequently DHA-S might act on the cervix by stimulating prostaglandin production. Phospholipid hydrolysis by phospholipase is in general considered a membrane event tightly coupled to the activation of cell surface receptors[20]. In summary: 1) the membrane phenomena occur in the fibroblasts exposed to DHA-S, 2) the effect of DHA-S on cervical ripening is due to unchanged DHA-S, 3) DHA-S is a water-soluble derivative of steroid, and 4) DHA-S is not metabolized to lipid-soluble DHA or oestrogens in the cervical fibroblasts we tested. Consideration of these data lead to the hypothesis that DHA-S interacts with membrane components and may act on the cervix by a membrane-mediated process.

Our results provide direct evidence that receptor components specific for DHA-S, but not for DHA or oestrone sulfate, are present in the plasma membrane of DHA-S-responsive cervical fibroblasts. The rate of membrane-associated binding of DHA-S is markedly rapid, and reaches equilibrium within 1–2 min. The values of apparent K_d and B_{max} are in close agreement with those of membrane-associated receptors for peptide hormones, but the rate of DHA-S uptake by cervical fibroblasts seems to be much higher, which conflicts with the assumption that steroid hormones enter cells by free diffusion[11]. The findings that no DHA is produced from DHA-S, and that no binding of oestrone sulfate occurs, could exclude the possibility that DHA-S binds to sulfatase in the plasma membrane. Some investigators have characterized membrane-binding sites with specificities for steroid hormones including oestradiol[21] and glucocorticoid[22].

Hodam et al.[23] have reported that another lipophilic extracellular signal molecule prostaglandin E_2, binds to uterine membranes. Our studies on binding kinetics are consistent with these observations. The nature and origin of these binding components and the manner in which they may contribute to the recognition and mediation of entry of DHA-S into the cell remain to be determined.

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1 Gant, N. F., Hutchinson, H. T., Siiteri, P. K., and MacDonald, P. C., Am. J. Obstet. Gynec. *111* (1971) 555.
2 Diczfalusy, E., Fedn Proc. *23* (1964) 791.
3 Madden, J. D., Siiteri, P. K., MacDonald, P. C., and Gant, N. F., Am. J. Obstet. Gynec. *125* (1976) 915.
4 Seron-Ferre, M., Taylor, N. F., Rotten, D., Koritnik, D. R., and Jaffe, R. B., J. clin. Endocr. Metab. *57* (1983) 1173.
5 Zuidema, L. J., Khan-Dawood, F., Dawood, M. Y., and Work, B. A., Am. J. Obstet. Gynec. *155* (1986) 1252.
6 Sasaki, K., Nakano, R., Kadota, Y., Iwao, M., Shima, K., and Soma, M., Br. J. Obstet. Gynec. *89* (1982) 195.
7 Danforth, D. N., Veis, A., Breen, M., Weinstein, H. G., Buckingham, J. C., and Manalo, P., Am. J. Obstet. Gynec. *120* (1974) 641.
8 Kleissl, H. P., van der Rest, M., Naftolin, F., Glorieux, F. H., and Deleon, A., Am. J. Obstet. Gynec. *130* (1978) 748.
9 Uldbjerg, N., Ekman, G., Malmstrom, A., Olsson, K., and Ulmsten, U., Am. J. Obstet. Gynec. *147* (1983) 662.
10 Rajabi, M. R., Dean, D. D., Beydoun, S. N., and Woessner, J. F. Jr, Am. J. Obstet. Gynec. *159* (1988) 971.
11 O'Malley, B. W., and Birnbaumer, L., in: Receptors and Hormone Action, vol. 2, p. 602. Academic Press, New York 1978.
12 Imai, A., Matsunami, K., Iida, K., and Tamaya, T., Biosci. Rep. *10* (1990) 47.
13 Evans, W. H., in: Laboratory Techniques in Biochemistry and Molecular Biology, vol. 7, p. 103. Elsevier, New York 1980.
14 Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. biol. Chem. *193* (1951) 265.
15 Kolenysi, R., and Woessner, J. F. Jr, Biol. Reprod. *42* (1990) 87.
16 Mochizuki, M., Honda, T., and Tojo, S., Int. J. Obstet. Gynec. *16* (1979) 248.
17 Ito, A., Sano, H., Ikeuchi, T., Sakyo, K., Hirakawa, S., and Mori, Y., Biochem. Med. *31* (1984) 257.
18 Thorburn, G. D., and Challis, J. R. G., Physiol. Rev. *59* (1979) 863.
19 Bleasdale, J. E., and Johnston, J. M., Rev. Perinat. Med. *5* (1984) 151.
20 Majerus, P. W., Ross, T. S., Cunningham, T. W., Caldwell, K. K., Jefferson, A. B., and Bansal, V. S., Cell *63* (1990) 459.
21 Pietras, R. J., and Szego, C. M., Nature *265* (1977) 69.
22 Suyemitsu, T., and Terayama, H., Endocrinology *96* (1975) 1499.
23 Hodam, J. R., Snabes, M. C., Kuehl, T. J., Jones, M. A., and Harper, M. J. K., J. molec. Endocr. *3* (1989) 33.

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Inhibition of IgM antibody-mediated aggregation of *Trypanosoma gambiense* in the presence of complement

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Abstract. This paper deals with the immune reaction between *Trypanosoma gambiense* and monoclonal IgM mouse antibody at equivalence with or without rabbit complement. Antibody-mediated trypanosome clumps formed in the absence of complement, and were readily dissociated by complement to become free. In the presence of complement, on the other hand, *T. gambiense* were not aggregated by the antibody. Free parasites adhered readily to cultured peritoneal macrophages. Complement-mediated dissociation of the clumped trypanosomes in the equivalence area released a large number of previously bound surface antigens. These antigens were capable of binding again to fresh IgM antibody. Experimental results further indicated that the complement system caused a functional alteration, changing the multivalent nature of the IgM antibody in the immune complex into a univalent one. This phenomenon is of great advantage to the infected host in clearing pathogens in vivo, as it allows more antibodies to attach to trypanosomes and subsequently initiate complement activity.
Key words. Trypanosoma gambiense; monoclonal IgM antibody; immune reaction; dissociation; immunophagocytosis.

Animals challenged with crude homogenates of blood trypanosomes were protected against the homologous strain of trypanosomes[1]. Antiserum passively transferred from immunized donors was also effective in protecting recipients against trypanosomes. These in vivo trials were initially suggested by in vitro observations on the immune reaction between the trypanosome, the antiserum and complement. In vitro, *T. gambiense* cells were aggregated by heat-inactivated antiserum[1]. The clumped trypanosome masses formed by heat-inactivated antiserum were dissociated by complement[2]. While the mechanism of the dissociation was obscure, it was reported recently that the dissociation of aggregates mediated by rabbit IgG antibody[3] or mouse mAb IgG 3 antibody[4] was associated with a functional change in the nature of the bivalent antibodies in the immune complex, which changed into univalent ones in the presence of complement.