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Uptake of dehydroepiandrosterone-3-sulfate by isolated trophoblasts from human term placenta, JEG-3, BeWo, Jar, BHK cells, and BHK cells transfected with human sterylsulfatase-cDNA

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Abstract

The human placenta lacks the enzyme 17α-hydroxylase/17-20-lyase, and is thus unable to convert cholesterol into estrogens. Therefore estrogen synthesis of trophoblast cells depends on the supply of precursors such as dehydroepiandrosterone-3-sulfate (DHEA-S) and 16α-hydroxy-dehydroepiandrosterone-3-sulfate by maternal and fetal blood. To investigate the cellular internalisation of these anionic hydrophilic precursors, the uptake of [^{3}H]-/[^{35}S]-DHEA-S and [^{3}H]-taurocholate by isolated cytotrophoblasts, cells of choriocarcinoma cell lines (JEG-3, BeWo, Jar), BHK and BHK cells transfected with human sterylsulfatase-cDNA (BHK-STS cells) was studied. Furthermore, the activity of sterylsulfatase of these cells in suspension and in corresponding cell homogenate was measured.

During the first 5 min of incubation with [^{3}H]-DHEA-S or [^{35}S]-DHEA-S, radioactivity of cytotrophoblasts increased significantly, while radioactivity of JEG-3, Jar, BHK and BHK-STS cells did not increase. Radioactivity of BeWo cells increased slightly. For all cell types, there was no significant difference for uptake of either substrate. During incubation with [^{3}H]-taurocholate, radioactivity of cytotrophoblasts did not increase. Sterylsulfatase activity of cytotrophoblast homogenate was significantly lower than that of cytotrophoblast suspension. Sterylsulfatase activity of BHK-STS, JEG-3 or BeWo cell homogenate was significantly higher than that of the corresponding cell suspension. In BHK and Jar cells sterylsulfatase activity was not detectable.

Cytotrophoblasts take up DHEA-S without prior hydrolysis. BHK, BHK-STS, JEG-3, and Jar cells do not take up and BeWo cells slowly take up DHEA-S. In cytotrophoblasts extracellular DHEA-S rapidly gains access to intracellular sterylsulfatase, while in choriocarcinoma and BHK-STS cells access of DHEA-S to sterylsulfatase is limited. Our results indicate, that uptake by cytotrophoblasts is mediated by a carrier which is not expressed in choriocarcinoma or BHK cells and which is different from the known taurocholate-transporting organic anion transporting polypetides.

1. Introduction

In primates, plasma concentrations of unconjugated estrone, estradiol, and estriol increases linearly with advancing gestation. After approximately week 9 of human pregnancy, the placenta becomes the main source of maternal estrogens. However, because this steroidogenic organ lacks the enzyme system 17α-hydroxylase/17-20-lyase, and thus is unable to convert

Abbreviations: taurocholate, N-(2-sulfoethyl)-5β-cholan-3α,7α,12α-triol-24-amide; DHEA-S, dehydroepiandrosterone-3-sulfate; 3β-sulfoxy-androst-5-ene-17-one; STS, sterylsulfatase; steryl-sulfate sulfohydrolase (EC 3.1.6.2).

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cholesterol into estrogen, its estrogen synthesis is highly dependent on the supply of C_{19} steroids for their conversion into estrogens. It was demonstrated that sulfated C_{19} steroids of fetal or maternal origin serve as precursors for the placental estrogen biosynthesis and that the resulting estrogens are transferred to the maternal blood (for review see [1–3]).

Dehydroepiandrosterone-3-sulfate (DHEA-S) of maternal and fetal origin contributes about equally to the placental formation of estrone and estradiol, while 16α-hydroxydehydroepiandrosterone-sulfate supplied by the fetus contributes to over 90% of placental estriol synthesis. Therefore, the concept of a functional fetal placental unit for estrogen synthesis was established, providing the basis for maternal estriol measurement to assess fetal well being, placental function and/or uteroplacental blood flow (for review see [2]).

The conversion of sulfurylated C_{19} steroid precursors, such as DHEA-S, to estrogens involves the action of four enzymes located predominantly intracellularly in the syncytiotrophoblast [4]. Therefore the substrates must enter the cells. The mechanism of their uptake by placental cells is unknown. Since the hydrophilic and anionic sulfate esters are repelled biophysically by the cell membrane, we hypothesized that a carrier is involved in their placental uptake. Several years ago, Schwenk et al. [5] had shown that the uptake of estrone sulfate into isolated rat hepatocytes is carrier mediated and energy dependent. For the human placenta it was speculated, that the sterylsulfatase may play a role in steroid sulfate uptake [2,6,7]. To investigate this hypothesis and the mechanism of DHEA-S uptake into the human placenta we have studied the uptake of [^{3}H]- and [^{35}S]- labeled DHEA-S by cytotrophoblasts, cells of choriocarcinoma cell lines, BHK cells and BHK cells transfected with human sterylsulfatase cDNA (BHK-STS cells) in suspension.

2. Materials and methods

2.1. Materials

Silicon oil AR 200 and AR 20 were obtained from Wacker Chemie (München, Germany) and DHEA-S and taurocholate sodium salt from Sigma Chemie (München, Germany). All other substances were obtained from Merck (Darmstadt, Germany).

2.1.1. Cell isolation and culture

The origin of enzymes, culture media and culture flasks has been specified previously [8,9]. The BHK and choriocarcinoma cell lines JEG-3, BeWo and Jar were obtained from the American Type Culture Collection. The BHK cell line transfected with human sterylsulfatase cDNA was kindly provided by Professor von Figura (University of Göttingen, Germany) [7].

2.1.2. Radiochemicals

[7-^{3}H(N)]-DHEA-S sodium salt, [^{3}H(G)]-taurocholic acid and [^{3}H]-water were purchased from NEN DuPont (Bad Homburg, Germany) and [^{35}S]-H_2SO_4 and [^{3}H]-inulin from Amersham (Braunschweig, Germany). [^{35}S]-DHEA-S was synthesized as described earlier [10].

2.2. Isolation of cells

Mononuclear human cytotrophoblasts were isolated and purified from term placenta obtained after spontaneous vaginal delivery or uncomplicated caesarea section as described earlier [8,11]. Usually more than 90% of the cells did not stain immunologically for vimetin but did stain for cytokeratin [12].

The cell lines were grown to confluency in 250 ml tissue culture flasks at 37°C in humidified 5% CO_2/95% air using DMEM with 5% fetal calf serum (BHK cells), or Ham's F12 with 15% fetal calf serum (JEG-3, BeWo, Jar cells) supplemented with 2.0 mM glutamine, 100 iU/ml penicillin and 100 μg/ml streptomycin. BHK-STS cells were grown like BHK but in addition the medium contained 5 μg/ml puromycin. Prior to experiments, the cells were trypsinized and resuspended in culture medium.

The viability of the cells, as assessed by trypan blue exclusion, exceeded 90%.

2.3. Transport studies

Cell suspensions obtained as described above, were centrifugated and resuspended to 1.5×10^6 cells/ml in cold transport buffer, containing 142.9 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$ and 1.8 mM $CaCl_2$, and kept on ice.

For transport studies aliquots of the cell suspension were stirred at 37°C for 20 min. Transport reaction was started by adding a 30-fold concentrated substrate solution consisting of varying concentrations of unlabelled substrate and trace amounts of [^{3}H] or [^{35}S]-labelled substrate. At the desired times, cells were separated from incubation medium by centrifugal filtration through a mixture of silicon oil AR 200 and AR 20 (density $\rho = 1.012$ g/cm^3) according to [13] with the following modifications: (1) the 3 mM KOH solution was omitted; (2) the volume of the silicon layer was increased to 150 μl; and (3) immediately after centrifugation, the vials were frozen in liquid nitrogen and cut 6 mm above the bottom. Radioactivity of the cell pellet was analysed in a LKB liquid scintillation coun-

ter. Measurements with each cell preparation were performed three to six times.

2.4. Estimation of extracellular and intracellular water space

Cells were incubated at 37°C for 5 min with trace amounts of [^{3}H]-inulin or for 10 min with trace amounts of [^{3}H]-water and centrifugated through silicon oil as described above.

2.5. Sterylsulfatase activity

Suspension: Aliquots of the cell suspensions were incubated with 10 μM [^{35}S]-DHEA-S as described above. The enzymatic reaction was stopped by adding charcoal suspended in a solution of 0.1 M citric acid and 0.1 M Na_2SO_4. The suspension was processed as described [10].

Homogenate: Cells were suspended in 100 mM Tris/acetate buffer pH 7.0 (3–5 × 10^6 cells/ml) and sonicated (5 pulses, 5 s each). Sterylsulfatase activity was determined with 10 μM [^{35}S]-DHEA-S as described earlier [8,10].

2.6. Cellular protein

Cellular protein was determined using a modification of the method described by Lowry et al. [14].

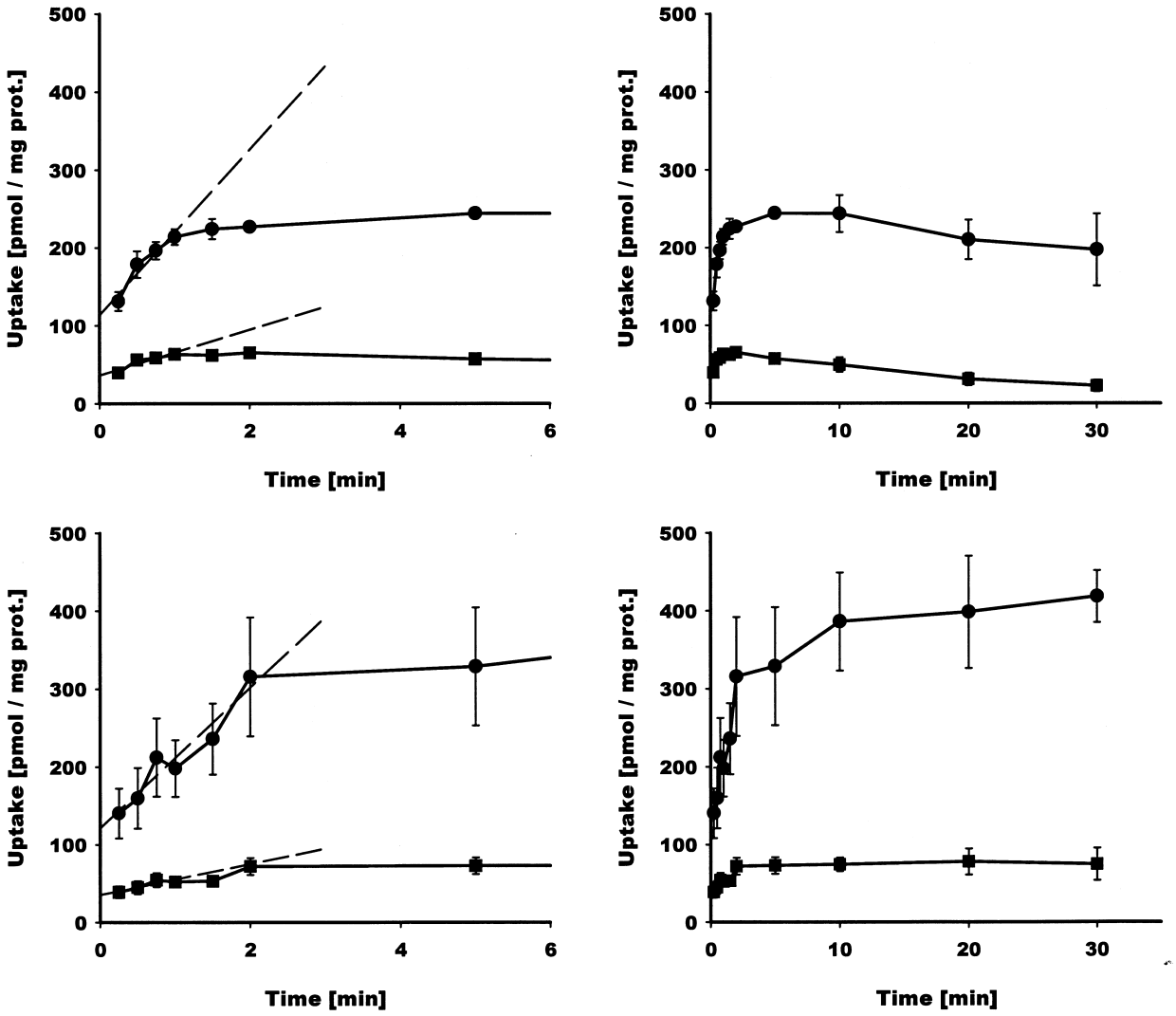

Fig. 1. Time course of DHEA-S uptake by cytotrophoblasts. Suspended cells (1.5 × 10^6 cells/ml) were incubated with 1 μM (■) or 5 μM (●) [^{3}H]-DHEA-S (below) or [^{35}S]- DHEA-S (above). At indicated times, aliquots were taken and cells centrifugated through silicone oil. The radioactivity of the pellet was measured, and the amount of DHEA-S, sedimented with the cells was calculated. Each point represents the arithmetic mean ± standard deviation of the values of 3 cell preparations with 3–6 estimations. Left: short term incubation; right: long term incubation. — — — — represents slope of initial uptake (v) after 0.25, 0.5, 0.75 and 1.0 min.

2.7. Statistics

The slope of initial uptake (v) was estimated by linear regression analysis of DHEA-S uptake after 0.25, 0.5, 0.75 and 1.0 min using the computer program SigmaPlot. To test for significance between data sets, double-sided t tests were performed.

3. Results

3.1. Uptake studies

3.1.1. DHEA-S

During the first 5 min of incubation with [^{3}H]-DHEA-S or [^{35}S]-DHEA-S cell-associated radioactivity of cytotrophoblasts increased (see Fig. 1). The slope calculated for the initial uptake using linear regression analysis was significantly different from 0 (see Table 1). There was no significant difference between the uptake of [^{3}H]-DHEA-S and [^{35}S]-DHEA-S. After 10 min, cell-associated radioactivity remained constant for [^{3}H]-DHEA-S and decreased for [^{35}S]-DHEA-S (see Fig. 1).

During the first 5 min of incubation with [^{3}H]-DHEA-S or [^{35}S]-DHEA-S cell-associated radioactivity of BHK cells (data not shown), BHK-STS (see Fig. 2), JEG-3 (see Fig. 3) and Jar cells (data not shown) did not increase. The calculated slope of initial uptake was not significantly different from zero (see Table 1). There was no significant difference between uptake of [^{3}H]-DHEA-S and [^{35}S]-DHEA-S. After 10 min the cell-associated radioactivity of BHK, JEG-3 and Jar cells remained constant for both substrates, while for [^{3}H]-DHEA-S the cell-associated radioactivity of BHK-STS cells increased (Fig. 2).

During the first 5 min of incubation with [^{3}H]-DHEA-S or [^{35}S]-DHEA-S cell-associated radioactivity of BeWo cells increased slightly. There was no significant difference between uptake of [^{3}H]-DHEA-S and [^{35}S]-DHEA-S (data not shown).

3.1.2. Taurocholate

During incubation with [^{3}H]-taurocholate for up to 30 min, cell-associated radioactivity cytotrophoblasts did not increase (Fig. 4). The calculated slope of initial uptake was not significantly different from zero (data not shown).

3.2. Sterylsulfatase activity

Specific sterylsulfatase activity of homogenated cytotrophoblasts was significantly lower ($p < 0.05$) than sterylsulfatase activity of cell suspension. Specific sterylsulfatase activity of homogenated BHK-STS and JEG-3 cells was significantly higher ($p < 0.01$) than sterylsulfatase activity of cell suspension. In BeWo cells specific sterylsulfatase activity of homogenate did not differ significantly from sterylsulfatase activity of cell suspension. In BHK and Jar cells no sterylsulfatase activity was detectable (see Table 2).

3.3. Extra- and intracellular water space

To correct the cell-associated DHEA-S for the amount of DHEA-S adherent to the sedimented cells with incubation medium and to calculate the hypothetical intracellular concentration of DHEA-S the extracellular (''inulin'') and total water space of the cells were determined. The extracellular volume of cytotrophoblasts and choriocarcinoma cells was similar (1.5–2.1 μl/(mg protein)) but appreciably lower (0.8–0.9 μl/(mg protein)) for BHK and BHK-STS cells (Table 3). The intracellular water space (total water space — extracellular ''inulin'' space) was approximately 4–5 μl/(mg protein) for cytotrophoblasts and BHK cells and about 6–7 μl/(mg protein) for choriocarcinoma cells.

Table 1
Slope of initial uptake (v) of DHEA-S by suspended cells. Values are arithmetic means (standard error) of the results of 3–4 experiments and are expressed in pmol/(mg protein x min)

	Substrate			
	[^{35}S]-DHEA-S		[^{3}H]-DHEA-S	
Cell type	1 μM	5 μM	1 μM	5 μM
Cytotrophoblasts	29.3 (4.8)[a]	106.4 (15.2)[a]	19.9 (7.0)[b]	90.2 (40.1)[b]
BHK-STS cells	2.1 (0.9)	17.5 (10.6)	1.5 (1.7)	3.7 (6.7)
BHK cells	1.2 (0.9)	14.2 (8.3)	−0.5 (1.4)	5.5 (8.1)
JEG-3 cells	1.6 (2.7)	−6.9 (8.2)	−0.7 (1.7)	−1.6 (13.2)
BeWo cells	0.5 (2.1)	15.6 (11.7)	3.0 (1.4)[b]	13.9 (10.5)
Jar cells	0.3 (2.0)	2.7 (9.8)	−0.7 (0.9)	0.1 (6.6)

[a] Parameter significant different from zero $p < 0.0001$.
[b] Parameter significant different from zero $p < 0.05$.

Table 2
Sterylsulfatase activity of suspended or homogenated cells. Values are arithmetic means (standard deviation) of the results of 3–4 experiments and are expressed in pmol/(mg protein × min)[a]

Cell type	Suspension	Homogenate
Cytotrophoblasts	201.0 (13.5)	149.9 (32.2)
BHK-STS cells	117.5 (10.2)	623.1 (98.3)
BHK cells	n.d.	n.d.
JEG-3 cells	45.8 (16.7)	171.4 (17.9)
BeWo cells	5.5 (5.5)	15.3 (7.2)
Jar cells	n.d.	n.d.

[a] n.d. = not detectable = < 3.

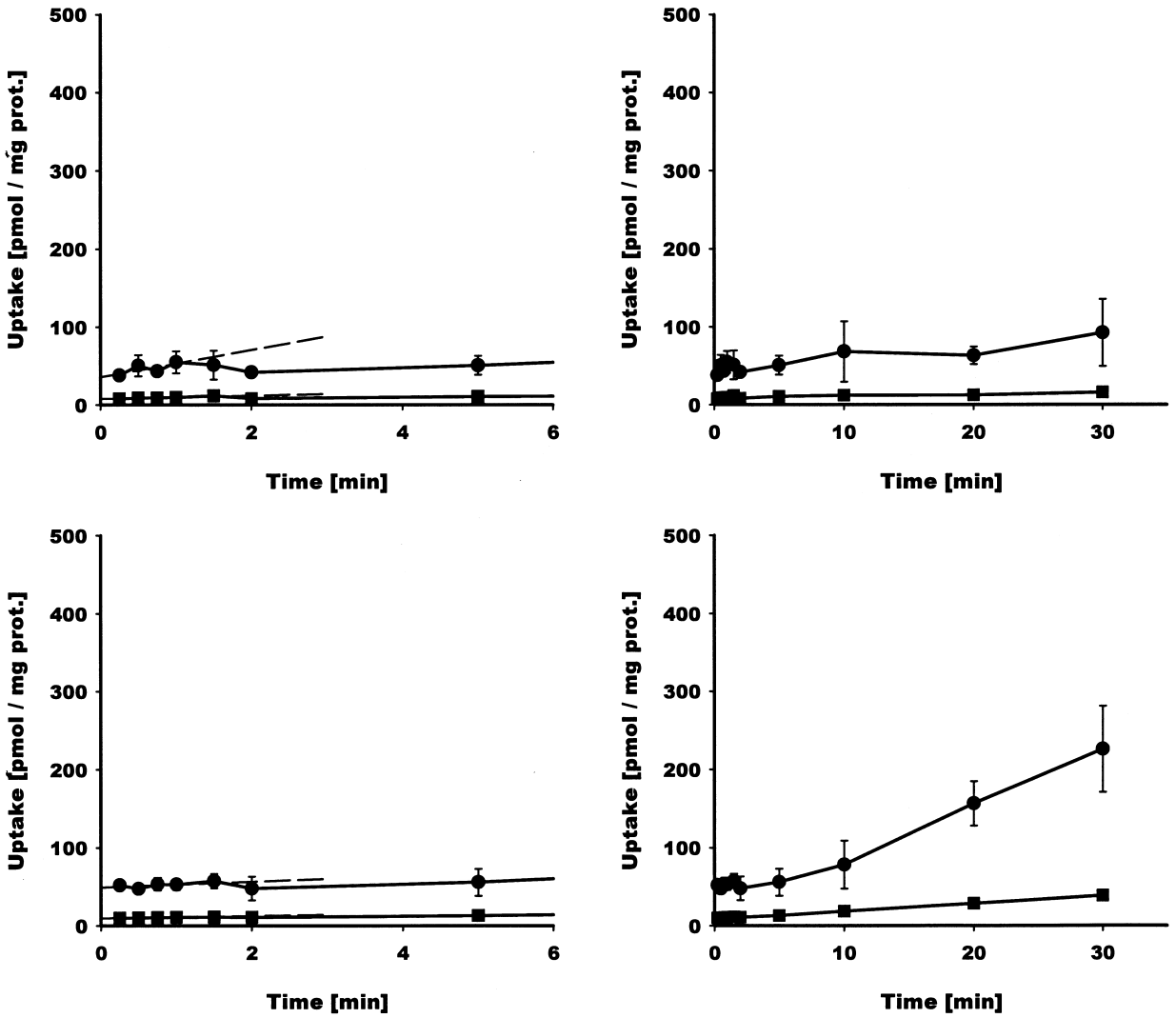

Fig. 2. Time course of DHEA-S uptake by BHK cells transfected with human placental STS cDNA (BHK-STS cells). Suspended cells (1.5×10^6 cells/ml) were incubated with 1 μM (■) or 5 μM (●) [^{3}H]-DHEA-S (below) or [^{35}S]- DHEA-S (above). At indicated times, aliquots were taken and cells centrifugated through silicone oil. The radioactivity of the pellet was measured, and the amount of DHEA-S, sedimented with the cells was calculated. Each point represents the arithmetic mean ± standard deviation of the values of 3–4 cell preparations with 3–6 estimations. Left: short term incubation; right: long term incubation. — — — — represents slope of initial uptake (v) after 0.25, 0.5, 0.75 and 1.0 min.

Table 3
Estimation of extra- and intracellular water space. Values are arithmetic means (standard deviation) of the results of 3–4 experiments and are expressed in μl/(mg protein)

Cell type	Total water space	Extracelluar ("inulin") space
Cytotrophoblasts	6.24 (0.65)	2.13 (0.38)
BHK-STS cells	6.26 (0.43)	0.83 (0.05)
BHK cells	5.35 (0.66)	0.92 (0.22)
JEG-3 cells	7.53 (0.18)	1.58 (0.14)
BeWo cells	9.00 (0.39)	1.89 (0.23)
Jar cells	7.71 (0.59)	1.51 (0.1)

4. Discussion

DHEA-S and 16α-OH-DHEA-S from the fetal and maternal circulation have to cross cellular membranes before intracellular sulfatase, 3β-hydroxy-steroiddehydrogenase and aromatase can gain access to their substrates for estrogen synthesis. The mechanism of uptake of steroid sulfate anions by cells is still unknown.

In the presented study, uptake rates of steroid sulfates were determined from the slope of the linear-regression analysis of multiple data points between 15 and 60 s in the time vs radioactivity diagram. The resulting *time 0 intercepts* were positive and nearly of similar length for all cells and both substrates. Only a

small part (<10%) of these positive *time 0 intercepts* is due to radioactivity of the substrate in the incubation medium adherent to the cells after centrifugation through silicone oil. The remaining part of *time 0 intercepts* probably represents substrate bound mainly nonspecifically to the cellular membrane. Nonspecific binding of numerous other amphipatic organic anions to cell membranes has been discussed previously (e.g. [13,15–18]).

Significant initial uptake rates of DHEA-S were observed only for isolated cytotrophoblasts and to a lesser extent for BeWo but not for JEG-3, Jar, BHK cells and BHK-STS cells (Table 1). These results may indicate, that DHEA-S uptake is mediated by a carrier, expressed in trophoblasts and to some extent by BeWo cells but not in JEG-3, Jar, BHK and BHK-STS cells. After 10 min of incubation cell-associated radioactivity of cytotrophoblasts remained constant for [^{3}H]-DHEA-S and decreased for [^{35}S]-DHEA-S (see Fig. 1). This decrease might be due to intracellular hydrolysis of DHEA-S and an efflux of free [^{35}S]-sulfate ions, presumably via the sulfate carrier [19], and a trans inhibition of DHEA-S influx by DHEA or DHEA metabolites. Cell-associated radioactivity of BHK-STS cells was constant up to 30 min for [^{35}S]-DHEA-S but increased after about 10 min for [^{3}H]-DHEA-S (Fig. 2). This increase might be due to extracellular hydrolysis of DHEA-S and an influx of uncharged [^{3}H]-DHEA due to its lipophilic property. The extracellular hydrolysis was thought to be cata-

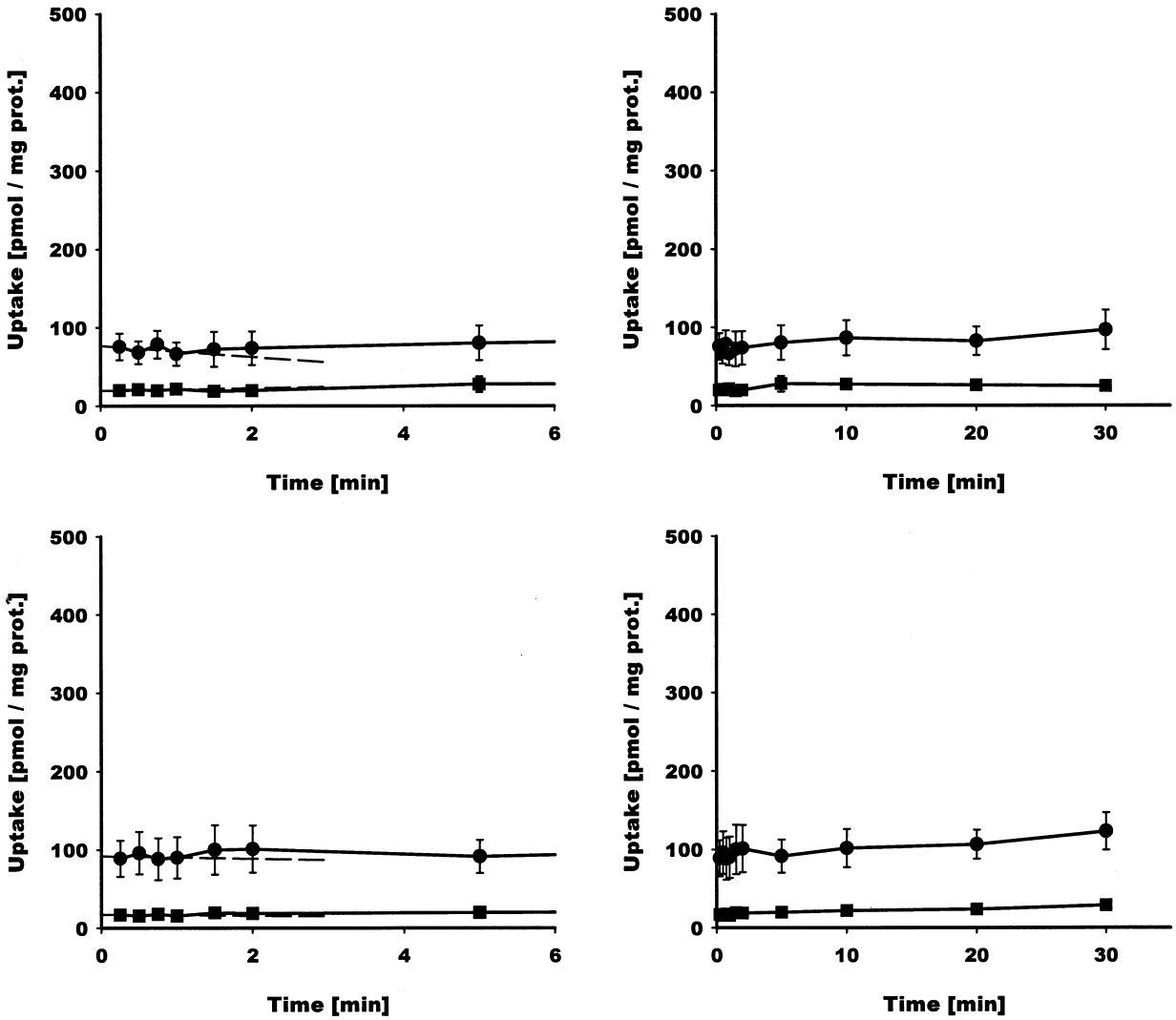

Fig. 3. Time course of DHEA-S uptake by JEG-3 cells. Suspended cells (1.5×10^6 cells/ml) were incubated with 1 μM (■) or 5 μM (●) [^{3}H]-DHEA-S (below) or [^{35}S]-DHEA-S (above). At indicated times, aliquots were taken and cells centrifugated through silicone oil. The radioactivity of the pellet was measured, and the amount of DHEA-S, sedimented with the cells was calculated. Each point represents the arithmetic mean ± standard deviation of the values of 4 cell preparations with 3–4 estimations. Left: short term incubation; right: long term incubation. — — — — represents slope of initial uptake (v) after 0.25, 0.5, 0.75 and 1.0 min.

lyzed by sulfatase released from damaged cells or by sulfatase located in the cellular plasma membrane (see below).

The hypothesis that DHEA-S uptake by trophoblasts is mediated by a carrier which is not expressed in the other cells tested, is supported by the following observations: Sterylsulfatase activity of whole cytotrophoblast in suspension was higher than sterylsulfatase activity of cytotrophoblast homogenated by ultrasonification. Sterylsulfatase activity of intact JEG-3 and BHK-STS cells in suspension was lower than sterylsulfatase activity of homogenates of JEG-3 and BHK-STS cells (Table 2). Obviously the intracellular sterylsulfatase of intact cytotrophoblasts can gain access to extracellular DHEA-S, after cellular uptake of the substrate via the carrier, while in JEG-3 and BHK-STS cells intracellular sterylsulfatase has no or only limited access to extracellular DHEA-S due to lack of the carrier. During incubation with 5 μM DHEA-S cytotrophoblast-associated DHEA-S increased from about 125 pmol/(mg prot.) at 0.25 min to about 250 pmol/(mg prot.) at 1 min. Relative to the calculated intracellular water space this reflects a 5–6-fold higher intracellular concentration of DHEA-S after 1 min. Whether this is due to an active transport or binding to intracellular membranes or binding proteins is unclear. In previous studies, the K_m-values of solubilized/purified sterylsulfatase were reported to be 25 μM [20], 1.7 μM [21] and 7.8 μM [22], and of microsomal (membrane bound) sterylsulfatase 100 μM [20] and 3.5 μM [10,21] with DHEA-S as the substrate. Thus, the higher sterylsulfatase activity of cytotrophoblasts in suspension compared to homogenated cytotrophoblasts could be explained by a higher intracellular substrate concentration. However, it could not be excluded, that the difference in sterylsulfatase activity between intact and homogenized cells may be due to stability of the enzyme or the dramatic modification of the enzyme environment which influence the enzyme activity. But for this explanation, one has to postulate a different behavior of sterylsulfatase from cytotrophoblasts compared to sterylsulfatase from JEG-3 and BHK-STS cells.

The sterylsulfatase consists of 562 amino acid residues [7,23]. From cDNA data, a hydrophobic domain was deduced which comprises two sequences that span the membrane in opposite directions, with a proline residue allowing the turn. The flanking regions, the N- and C-terminal domain may be exposed to the luminal side of the endoplasmatic reticulum, consist of 161 and 346 residues, respectively, and contain one glycosylated asparagine residue [7]. The C-terminal domain seems to contain the substrate binding site including the histidine residue which may be involved in catalyzing the sulfate ester hydrolysis [6,24]. Thus, after vesicular transport of sterylsulfatase-containing membranes from the endoplasmatic reticulum to the cell membrane, the catalyzing site of sterylsulfatase is exposed extracellularly. This hypothesis is supported by the findings that small amounts of sterylsulfatase are located in plasma membranes and along the endocytotic pathway of trophoblast [4,25]. Due to these findings it was speculated earlier that this enzyme may play a role in steroid sulfate uptake [2,6].

Using [^{35}S]- and [^{3}H]-labelled DHEA-S we have shown that DHEA-S is taken up by cytotrophoblasts

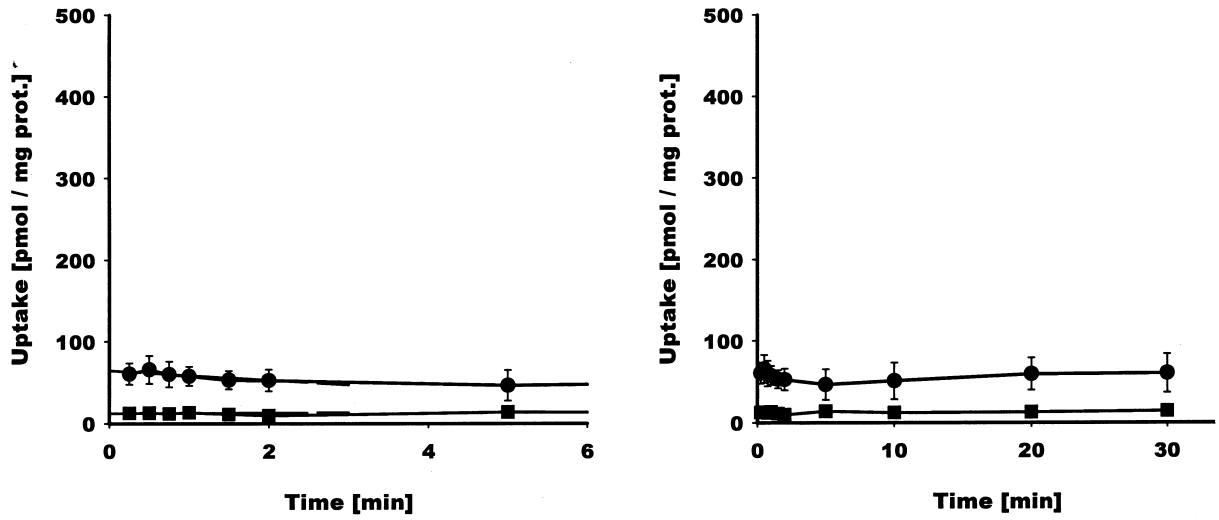

Fig. 4. Time course of taurocholate uptake by cytotrophoblasts. Suspended cells (1.5×10^6 cells/ml) were incubated with 1 μM (■) or 5 μM (●) [^{3}H]-taurocholate. At indicated times, aliquots were taken and cells centrifugated through silicone oil. The radioactivity of the pellet was measured, and the amount of taurocholate, sedimented with the cells was calculated. Each point represents the arithmetic mean ± standard deviation of the values of 4 cell preparations with 3–6 estimations. — — — — represents slope of initial uptake (v) after 0.25, 0.5, 0.75 and 1.0 min.

very likely without prior hydrolysis. BHK-STS cells did not take up DHEA-S. These observations indicate, that the cellular uptake of DHEA-S is not mediated by the enzyme sterylsulfatase. However, it could not be excluded, that there is a difference in structure and/or cellular orientation of sterylsulfatase expressed by trophoblasts or BHK-STS cells.

Recently, multispecific organic anion transporting polypeptides have been cloned from rat (oatp1) and human (OATP) liver [26,27], rat brain (oatp2) [28] and retina (oatp3) [29] and expressed in *Xenopus laevis* oocytes. Their substrates include sulfobromophthalein, bile acids, estrone-3-sulfate, DHEA-S, ouabain and other neutral steroids, as well as certain amphipathic organic anions [29–33]. These organic anion transporting polypeptides seem to be responsible for the uptake of estrone-3-sulfate by rat hepatocytes, as reported earlier by Schwenk et al. [16] and confirmed by Hassen et al. [34]. The fact, that isolated cytotrophoblasts did not take up taurocholate (Fig. 4) and uptake of taurocholate by basal plasma membrane vesicles of human syncytiotrophoblasts was not inhibited by estrone-3-sulfate [35] suggests, that DHEA-S uptake by these cells is mediated by a carrier protein different from these known multispecific organic anion transporting polypeptides and also different from the presumed carrier for bile acids of basal and apical membrane of human syncytiotrophoblast [36,37]. Further characteristics, such as substrate specificity of the presumptive DHEA-S carrier remain to be investigated. Since DHEA-S and other 3-O-sulfoconjugated steroids are synthesized by intracellular sulfotransferases of many cells (e.g. [2,38–41]) and utilized by many other non-placental human cells (e.g. [42–44]), one may speculate whether this carrier is also involved in the export/import of DHEA-S and other 3-O-sulfoconjugated steroids by these cells.

In conclusion, our results indicate, that uptake of DHEA-S by cytotrophoblasts is most likely mediated by a carrier which is not or only weakly expressed in choriocarcinoma and BHK cells and is different from the enzyme sterylsulfatase and known taurocholate transporting organic anion transporting polypetides.

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