

Modification of OATP2B1-Mediated Transport by Steroid Hormones

Markus Grube, Kathleen Köck, Susanne Karner, Sebastian Reuther, Christoph A. Ritter, Gabriele Jedlitschky, and Heyo K. Kroemer

Research Center of Pharmacology and Experimental Therapeutics, Department of Pharmacology (M.G., K.K., S.K., S.R., G.J., H.K.K.) and Department of Pharmacy (C.A.R.), Ernst Moritz Arndt University, Greifswald, Germany

Received May 8, 2006; accepted August 14, 2006

ABSTRACT

The family of the organic anion transporting polypeptides forms an increasing group of uptake transport proteins with a wide substrate spectrum. Although the expression of some members of this group, such as organic anion transporting polypeptide (OATP)-A or C, is limited to special tissues (such as liver or brain), the organic anion transporting polypeptide 2B1 (OATP-B/SLCO2B1) is expressed in many organs, including liver, placenta, mammary gland, brain, and intestine. However, little is known about its function in those tissues because only a limited number of compounds, such as dehydroepiandrosterone-sulfate (DHEAS) and estrone-3-sulfate (E3S), have been characterized as OATP2B1 substrates. To further elucidate the role of OATP2B1 on steroid transport, we examined the influence of steroid hormones on OATP2B1-mediated E3S and DHEAS uptake using OATP2B1-overexpressing Madin-Darby canine kidney II cells. We identified unconjugated androgens (e.g., tes-

tosterone) as potent inhibitors for OATP2B1. In contrast, gestagens such as progesterone enhanced E3S uptake in a concentration-dependent manner to up to 300% of the control, accompanied by a significant decrease in the OATP2B1 K_m value for E3S (control, $K_m = 14 \mu\text{M}$; in the presence of $31.6 \mu\text{M}$ progesterone, $K_m = 3.6 \mu\text{M}$). Moreover, we demonstrated that testosterone and progesterone are not substrates of OATP2B1, indicating an allosteric mechanism for the observed effects. Furthermore, we showed that progesterone enhances the OATP2B1-dependent pregnenolone sulfate transport. Taken together, the results indicate functional modification of OATP2B1-mediated E3S and DHEAS as well as pregnenolone sulfate transport through steroid hormones such as progesterone. These effects can have physiological consequences for the organ-specific uptake of steroids.

Organic anionic polypeptide transporters (OATPs) belong to the solute carrier [SLC 21; new classification SLCO (Hagenbuch and Meier, 2004)] family, a group of multispecific uptake proteins that mediate the sodium-independent transport of a wide variety of endogenous and xenobiotic compounds, which are generally large amphiphatic molecules and are mainly bound to albumin under physiological conditions. So far, 11 members of this family have been identified in humans: OATP1A2 (OATP-A), OATP2B1 (OATP-B), OATP1B1 (OATP-C), OATP3A1 (OATP-D), OATP4A1 (OATP-E), OATP1C1 (OATP-F), OATP4C1 (OATP-H), OATP5A1 (OATP-J), OATP6A1 (OATP-I), OATP1B3

(OATP8), and OATP2A1 (PGT) (Hagenbuch and Meier, 2004). These transporters exhibit homology among each other with an overall amino acid identity of 31 to 82%. They demonstrate a high variability in tissue distribution as well as in functional characteristics (reviewed in Hagenbuch and Meier, 2003; Hagenbuch and Meier, 2004). OATP1B1, for example, has a broad substrate specificity, which comprises bile salts, prostaglandins, and steroid conjugates, but is exclusively localized in liver, where its main function might be the elimination of compounds (Tamai et al., 2000; Cui et al., 2001; Kullak-Ublick et al., 2001). On the other hand, OATP2B1 has, as known so far, a narrow substrate spectrum, which includes estrone-3-sulfate (E3S), dehydroepiandrosterone-sulfate (DHEAS), and bromosulphophthalein, but shows a broad tissue distribution (Tamai et al., 2000; Pizzagalli et al., 2003). OATP2B1 expression is demonstrated in many organs such as liver, placenta, mammary gland, brain, and small intestine (Kullak-Ublick et al., 2001). In the latter, an apical expression in epithelial cells and a pH-

This work was supported by grants from the Deutsche Forschungsgemeinschaft (SFB/TR 19-04), the Deutsche Krebsstiftung (grant 502735), and the German Federal Ministry of Education and Research (NBL3 program, reference 01 ZZ 0103).

M.G. and K.K. contributed equally to this work.

Article, publication date, and citation information can be found at <http://molpharm.aspetjournals.org>.
doi:10.1124/mol.106.026450.

ABBREVIATIONS: OATP, organic anionic polypeptide transporter; E3S, estrone-3-sulfate; DHEAS, dehydroepiandrosterone-sulfate; MDCK, Madin-Darby canine kidney; DMSO, dimethyl sulfoxide; P-gp, P-glycoprotein.

sensitive transport with higher activity at acidic pH (transport of pravastatin) was observed (Kobayashi et al., 2003). Satoh et al. (2005), however, could not demonstrate pH sensitivity of the E3S transport, which might be because of the different cell types used. It is noteworthy that OATP2B1, despite its apical localization in the gastrointestinal tract, shows a basal/basolateral expression in other tissues. For example, OATP2B1 has been identified as a carrier in human mammary gland with localization in myoepithelial tissue, surrounding ductal epithelial cells, and in the basal membrane of the human placental syncytiotrophoblast, where it might be relevant for supply of steroid hormone precursors such as DHEAS to the fetoplacental unit (St-Pierre et al., 2002; Pizzagalli et al., 2003; Ugele et al., 2003). DHEAS, the transport form of DHEA, represents the major circulating steroid in human blood, reaching concentrations up to 10 μ M (Baulieu, 1996). It is secreted predominantly by the adrenal cortex and serves as the principal conjugated prohormone for the biosynthesis of both estrogenic and androgenic steroids in peripheral tissues. It acts, in addition, as a neuroactive steroid in brain where it can also be synthesized (Baulieu and Robel, 1998). DHEAS has been identified as substrate for several members of the OATP family, particularly OATP1A2, which is mainly expressed in brain (Kullak-Ublick et al., 1998), the liver-specific OATP1B1 (Konig et al., 2000a), and OATP1B3 (Konig et al., 2000b) as well as for OATP2B1, with its wide tissue distribution (Kullak-Ublick et al., 2001).

To investigate the role of OATP2B1 in transport and supply of steroid hormone metabolites and precursors, we studied the effects of steroid hormones on OATP2B1 transport activity with regard to E3S and DHEAS uptake. In this article, we describe for the first time a stimulatory effect of progesterone on E3S and DHEAS uptake and characterize its kinetic parameters. Moreover, we identify pregnenolone sulfate as a new substrate for OATP2B1.

Materials and Methods

Cell Culture and Substances. Madin-Darby canine kidney (MDCKII) cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 2 mM L-glutamine, 1% minimal essential medium nonessential amino acids, and penicillin/streptomycin (0.5 U/ml and 150 μ g/ml). Cells were incubated at 37°C in an atmosphere containing 5% CO₂. Cells were incubated with 2.5 mM sodium butyrate 24 h before transport experiments to enhance the expression of the recombinant protein.

Radiolabeled substances were obtained as indicated in the respective section. All other substances used for transport studies were obtained from Sigma-Aldrich Chemie GmbH (Munich, Germany).

Cloning and Transfection. The full-length OATP2B1 cDNA was amplified using reverse-transcribed RNA from human placenta (forward primer, 5'-agctcactgcactcagcagtcagtg-3'; reverse primer, 5'-aaag-gactcagaggaggtactgctgtggtgc-3') and cloned into the mammalian expression vector pcDNA3.1/hygro (Invitrogen, Carlsbad, CA). After verifying the sequence by cycle sequencing and matching against the reference sequence (accession number AB026256.1), MDCKII cells were transfected with this construct using the FuGENE 6 Transfection Reagent (Roche, Penzberg, Germany). Cells were selected for antibiotic resistance using 0.6 mg/ml hygromycin B (Invitrogen).

Immunofluorescence Microscopy. Protein localization was investigated by confocal laser scanning immunofluorescence microscopy. For OATP2B1 a polyclonal antibody described before was used (rabbit, dilution 1:200) (Grube et al., 2005). After washing with phosphate-buffered saline, the coverslips were incubated for 1 h

with Alexa Fluor 488-labeled IgG (anti-rabbit IgG). Staining of nuclei was performed using a 1:2000 dilution of TOTO-3-iodide in DAKO Fluorescent Mounting Medium (Dako North America, Inc., Carpinteria, CA). The secondary antibodies as well as the TOTO dye were purchased from Invitrogen.

Immunoblot Analysis. Crude membrane fractions were loaded onto a 7.5% sodium dodecylsulfate-polyacrylamide gel after incubation in sample buffer at 95°C for 10 min. Immunoblotting was performed using a tank blotting system (Bio-Rad Laboratories, Hercules, CA) and an enhanced chemiluminescence detection system (GE Healthcare, Freiburg, Germany). The OATP2B1 primary antibody was diluted in Tris-buffered saline containing 0.05% Tween 20 and 5% bovine serum albumin. A secondary horseradish peroxidase-conjugated goat anti-rabbit antibody (BioRad) was used at a 1:2000 dilution.

Transport Studies. For characterization of OATP2B1 transfection, the respective cells were incubated with [³H]E3S (specific activity, 50 Ci/mmol) and [³H]DHEAS (specific activity, 60 Ci/mmol; both from Hartmann Analytic, Braunschweig, Germany) for 5 or 10 min, respectively, washed four times with ice-cold phosphate-buffered saline, and lysed with 0.2% SDS. An aliquot was mixed with 4 ml of scintillation cocktail (Rotiszint; Roth, Karlsruhe, Germany) and measured in a scintillation beta-counter (type 1409; PerkinElmer Wallac, Turku, Finland). For E3S uptake inhibition experiments as well as for transport studies with [³H]testosterone, [³H]progesterone, and [³H]pregnenolone sulfate (all 50 Ci/mmol; Biotrend, Köln, Germany), cells were seeded out in 24-well dishes, cultured to confluence, and incubated for indicated times with the respective substance at 37°C. Rates of transport are given in moles \times milligrams of protein⁻¹ \times minutes⁻¹ or as ratio of control. All transport studies were performed using an incubation buffer containing 140 mM NaCl₂, 5 mM KCl, 1 mM KH₂PO₄, 1.2 mM MgSO₄, 1.5 mM CaCl₂, 5 mM glucose, and 12.5 mM HEPES, pH 7.3.

Statistical Methods. Values are represented as means \pm S.D. Graphs and calculation were in general prepared using Excel (Microsoft Corp., Redmond, WA) or Prism software 3.0 (GraphPad Software, San Diego, CA) software. Student's *t* test was performed to determine statistical significance. Differences were considered significant at *p* < 0.05. The EC₅₀ values were calculated by nonlinear regression from a sigmoidal dose-response curve (variable slope, bottom value \geq 0) using GraphPad. *K_m* and *V_{max}* values were also calculated by nonlinear regression using the equation: $Y = (V_{\max} \cdot X)/(K_m + X)$ by GraphPad.

Results

Characterization of the OATP2B1-Overexpressing Cells. Characterization was performed by Western blot and immunofluorescence technique. Therefore, OATP2B1-transfected MDCKII cells and nontransfected cells were cultured until confluence and treated with 2.5 mM sodium butyrate for 24 h. For Western blotting, cells were harvested and crude membrane fractions were obtained. After gel electrophoresis, proteins were blotted onto a nitrocellulose membrane, and OATP2B1 expression was detected by antibody staining. The OATP2B1-transfected cells exhibited a strong band at approximately 84 kDa, which corresponds to the published molecular mass of OATP2B1 (St-Pierre et al., 2002) compared with nontransfected MDCKII cells. To verify this result and to determine the localization of OATP2B1, immunofluorescence staining was performed. The OATP2B1 expression was restricted to the plasma membrane of the transfected cells (data not shown).

E3S and DHEAS Uptake Studies. As a first screening approach, OATP2B1-transfected cells were incubated with tritium-labeled E3S and DHEAS in the presence of various steroids. Substances such as estrone, estriol, β -estradiol, mifepristone, testosterone, and pregnenolone sulfate significantly

inhibited E3S uptake. In contrast, steroids such as hydroxyprogesterone, pregnenolone, and progesterone enhanced the E3S uptake depending on their concentration (Table 1). For the uptake of DHEAS, similar results were observed; however, the inhibitory effects were less intense, whereas the inducing effects were much higher compared with the E3S results (Table 2). The inhibition/stimulation the compounds exerted on E3S uptake were similar to the effects on DHEAS transport. Interestingly, divergent results were obtained for estrone: E3S uptake was inhibited (68% of control for 100 μ M E3S), whereas transport of DHEAS was induced up to 280% of control by estrone concentration of 10 and 100 μ M (288 and 283%).

To further characterize the inhibitory effects of testosterone and mifepristone as well as the stimulatory effect of progesterone on E3S and DHEAS uptake mediated by OATP2B1, the concentration of the half-maximal effect (EC_{50}) was determined. For the inhibition of testosterone, EC_{50} concentrations of 21.2 ± 5.1 μ M for E3S and 15.4 ± 1.3 μ M for DHEAS uptake were calculated; for mifepristone, the respective concentrations were 4.7 ± 0.7 μ M for E3S and 2.2 ± 0.8 μ M for DHEAS transport. The observed maximal inhibition of E3S and DHEAS uptake by testosterone and mifepristone was approximately 75% of control for both substances. In contrast, progesterone induces the uptake of E3S to a maximum of 200% and for DHEAS to 400% of control. These stimulatory effects show half-maximal concentrations of 1.5 ± 0.6 μ M for E3S and 4.9 ± 1.5 μ M for DHEAS (EC_{50} values are given as mean \pm S.D. for three independent experiments). The maximal effects were obtained for a progesterone concentrations between 10 and 30 μ M, whereas higher concentrations lead to decreased induction (Fig. 1).

TABLE 1

Effect of different steroids on the OATP2B1-mediated E3S uptake
OATP2B1-transfected MDCKII cells were incubated with [3 H]E3S (1 μ Ci/ml; 1 μ M) for 5 min in the presence of indicated compounds ($n = 3$).

Substance	Concentration μ M	% of Control (Mean \pm S.D.)
Control		100
Aldosterone	100	110 \pm 11
Aldosterone	10	129 \pm 15
Androsterone	100	145 \pm 20*
Androsterone	10	105 \pm 9
Cortisone	100	107 \pm 13
Cortisone	10	125 \pm 15
Estrone	100	68 \pm 8*
Estrone	10	87 \pm 7
Estriol	100	43 \pm 10*
Estriol	10	96 \pm 5
β -Estradiol	10	35 \pm 4*
Hydrocortisol	100	83 \pm 15
Hydrocortisol	10	92 \pm 7
Hydroxyprogesterone	10	168 \pm 12*
Mifepristone	100	7 \pm 1*
Mifepristone	10	26 \pm 4*
Pregnenolone	50	129 \pm 4
Pregnenolone	10	171 \pm 27*
Progesterone	100	66 \pm 5
Progesterone	10	186 \pm 14*
Testosterone	100	27 \pm 4*
Testosterone	10	84 \pm 8
Conjugates		
Estrone-3-sulfate	10	43 \pm 5*
Estradiol-glucuronide	10	106 \pm 3
DHEAS	10	70 \pm 2*
Pregnenolone sulfate	10	28 \pm 7*
Pregnenolone acetate	10	83 \pm 15

* Significant vs. control for $P < 0.05$.

Transport of Labeled Progesterone and Testosterone. To determine whether the interaction of testosterone and progesterone with the OATP2B1-mediated uptake of E3S and DHEAS is caused by a direct competition, OATP2B1-mediated transport of these compounds was tested. Therefore, transfected and nontransfected cells were incubated with tritium-labeled progesterone, testosterone, E3S, and DHEAS. As shown in Table 3, E3S and DHEAS were taken up in a significantly higher amount into transfected cells compared with control cells (11.1 ± 1.1 to 301 ± 33 fmol/mg/min for E3S and 11.4 ± 1.9 to 36.3 ± 3.5 fmol/mg/min for DHEAS). However, no significant difference in the uptake of testosterone were observed between OATP2B1-transfected and control cells. Moreover, for progesterone an enhanced uptake into nontransfected cells was observed. Next, we tested whether E3S or DHEAS stimulated the uptake of tritium-labeled progesterone. Although progesterone had a stimulatory action upon E3S and DHEAS uptake, such reciprocal action was missing for progesterone uptake by E3S or DHEAS (data not shown).

Further experiments were carried out to investigate the stimulatory effect of progesterone on OATP2B1-mediated DHEAS uptake. OATP2B1-transfected cells were incubated with [3 H]DHEAS and different progesterone concentrations in the presence of DMSO (0.1%) and 1 and 10 μ M mifepristone (Fig. 2). The calculation of the EC_{50} revealed values of 2.8 ± 1.5 μ M for the DMSO control, 5.3 ± 2.9 μ M for 1 μ M mifepristone, and 2.4 ± 0.4 μ M for 10 μ M mifepristone (EC_{50} values are given as mean \pm S.D. for three independent experiments). However, the maximal induction effect decreases from 500% (control) to 144% in the presence of 10 μ M mifepristone, indicating a noncompetitive interaction of progesterone and mifepristone.

Kinetic Studies. Additional experiments were carried out to analyze the interaction type of E3S transport and progesterone. Transfected cells were incubated with different concentra-

TABLE 2

Effect of different steroids on the OATP2B1-mediated DHEAS uptake
OATP2B1-transfected MDCKII cells were incubated with [3 H]DHEAS (1 μ Ci/ml) for 10 min in the presence of indicated compounds ($n = 3$).

Substance	Concentration μ M	% of Control (mean \pm S.D.)
Control		100 \pm 3
Androsterone	100	121 \pm 5
Androsterone	10	159 \pm 27*
Estrone	100	283 \pm 43*
Estrone	10	288 \pm 57*
Estriol	100	78 \pm 13
Estriol	10	108 \pm 8
β -Estradiol	10	158 \pm 15*
Hydrocortisol	100	158 \pm 35
Hydrocortisol	10	93 \pm 11
Hydroxyprogesterone	100	241 \pm 6*
Hydroxyprogesterone	10	209 \pm 13*
Mifepristone	100	55 \pm 15*
Mifepristone	10	69 \pm 10*
Pregnenolone	50	179 \pm 14*
Pregnenolone	10	184 \pm 12*
Progesterone	100	303 \pm 36*
Progesterone	10	346 \pm 3*
Testosterone	100	55 \pm 4*
Testosterone	10	75 \pm 6*
Conjugates		
Estrone-3-sulfate	100	64 \pm 7*
Estrone-3-sulfate	10	92 \pm 10
Estradiol-glucuronide	10	95 \pm 13
Pregnenolone sulfate	100	43 \pm 7*

* Significant vs. control for $P < 0.05$.

tions of [^3H]E3S (0.1 to 31.6 μM) in the presence or absence of progesterone (1–100 μM). Results are displayed as a Lineweaver-Burk plot (Fig. 3A) and Eadie-Hofstee plot (Fig. 3B). K_m and V_{\max} values were calculated by nonlinear regression in dependence of the progesterone concentrations (Fig. 3C). Up to progesterone concentrations of 31.6 μM , the affinity of OATP2B1 toward E3S increases 4-fold from $K_m = 14 \mu\text{M}$ (DMSO control) to $K_m = 3.6 \mu\text{M}$ (31.6 μM progesterone), whereas higher progesterone concentrations ($K_m = 7.2 \mu\text{M}$ for 100 μM progesterone) resulted again in a decrease. In contrast, the maximal velocity of E3S uptake (V_{\max}) is decreasing from 169 (control) to 106 pmol/min/mg (for 100 μM progesterone).

OATP2B1 and Pregnenolone Sulfate. Pregnenolone sulfate (10 μM) significantly inhibited E3S and DHEAS transport to 28 and 43% of the control, respectively (Tables 1 and 2); moreover, an EC_{50} value of $3.5 \pm 1.5 \mu\text{M}$ was calculated for E3S uptake (Fig. 4A). To determine whether pregnenolone sulfate is like other sulfates subject to OATP2B1-mediated transport, pregnenolone sulfate uptake experiments were performed in transfected and non-transfected cells. Incubation with pregnenolone sulfate

revealed a time-dependent OATP2B1-mediated uptake. This transport was further stimulated by the addition of 10 μM progesterone, whereas progesterone had no effect on pregnenolone sulfate uptake into nontransfected cells (Fig. 4, B and C). To further characterize the stimulatory effect of progesterone on pregnenolone sulfate uptake, the concentration dependence was evaluated over a concentration range of 1.6 to 100 μM . Although progesterone had no effect on MDCKII cells, increasing concentrations of progesterone stimulated pregnenolone sulfate uptake with an EC_{50} value of 5.18 μM .

Discussion

OATP2B1 (formerly known as OATP-B) is a member of the OATP family and is widely expressed in human tissues. For example, OATP2B1 expression was demonstrated in the basolateral hepatocyte membrane, the basal membrane of the placental syncytiotrophoblast, the apical membrane of the enterocytes, and in myoepithelial cells of the mammary gland (Konig et al., 2000a; St-Pierre et al., 2002; Kobayashi et al., 2003; Pizzagalli et al., 2003). In contrast to other OATPs, such as

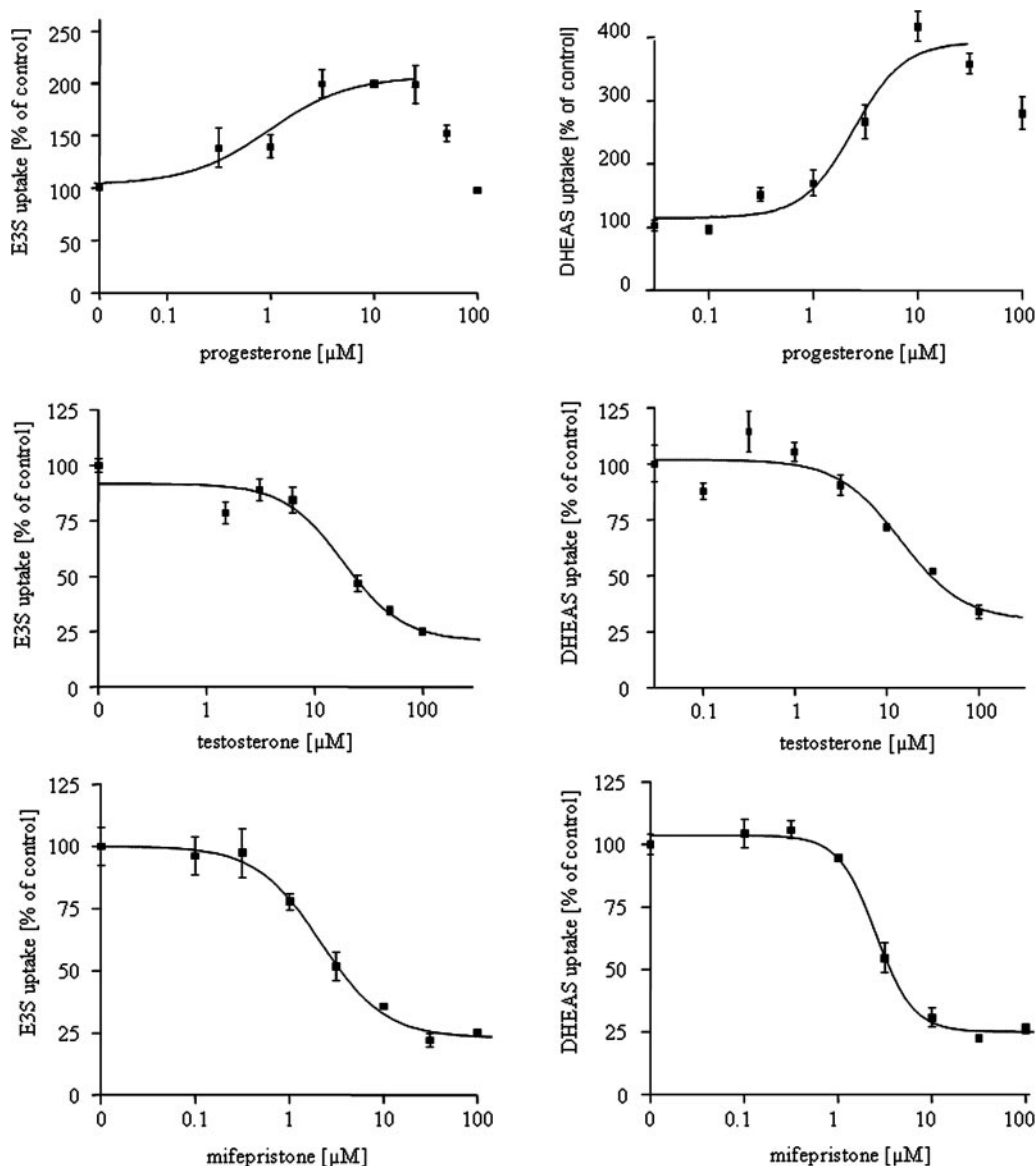


Fig. 1. Determination of the half-maximal concentration of the progesterone, testosterone, and mifepristone effect on OATP2B1-mediated E3S and DHEAS uptake. Cells were incubated for 10 min with [^3H]DHEAS (1 $\mu\text{Ci}/\text{ml}$; 1 μM) or 5 min with [^3H]E3S (1 $\mu\text{Ci}/\text{ml}$; 1 μM) in the presence of increasing concentrations of steroid hormones. Uptake is shown as percentage of DMSO control (mean \pm S.D.; $n = 3$ for a representative experiment).

OATP1B1 (OATP-C), which is described as a liver-specific uptake system for various glucuronide- and sulfate-conjugates, the spectrum of identified substrates of OATP2B1 mainly consists of sulfate-conjugates such as DHEAS and E3S (Tamai et al., 2001). Because of these transport properties and its localization in tissues such as the placenta or the mammary gland, which are involved in the steroid synthesis, OATP2B1 was discussed to be responsible for the uptake of precursor molecules such as DHEAS for the steroid hormone synthesis (Ugele et al., 2003). Moreover, E3S, which is the best characterized substrate of OATP2B1, seems not only to be a biologically inactive elimination product of estrogen but also to serve as an inactive pool for this hormone (Santner et al., 1986; Miki et al., 2002). For our inhibition experiments we used both E3S and DHEAS in a concentration of 1 μ M, which is in the range of the published K_m values of OATP2B1 for both substances (Tamai et al., 2001; Pizzagalli et al., 2003); moreover, DHEAS concentrations in a low micromolar range are also achieved under physiological conditions (Skalba et al., 2003). Taken together, an interaction of steroid hormones with the transport activity of OATP2B1 may be important. However, little is known about the interaction of OATP2B1 with steroid hormones such as gestagens, estrogens, and androgens. In the present study, we therefore investigated the interaction of OATP2B1-mediated transport with these compounds.

In a first screening approach using an OATP2B1-overexpressing cell system, we were able to show significant inhibition of E3S and DHEAS uptake by steroid hormones or

related compounds such as testosterone or mifepristone (Tables 1 and 2), whereas other steroids such as progesterone and pregnenolone demonstrated stimulatory effects on the uptake of both E3S and DHEAS. In the case of mifepristone and testosterone, the inhibitory effects were concentration-dependent with EC_{50} values of 4.7 μ M (2.2 μ M) and 21.2 μ M (15.4 μ M) for E3S (DHEAS), respectively. For both substrates used, the highest stimulatory effect of progesterone was observed for concentrations of around 10 μ M, whereas higher progesterone concentrations were only slightly inductive or even inhibitory for DHEAS and E3S uptake. The mifepristone EC_{50} values are in the range of pharmacologically relevant levels (Sarkar, 2002). In contrast, the EC_{50} values for progesterone and testosterone are very high compared with systemic concentrations [physiological progester-

TABLE 3

Uptake of [3 H]E3S (20 nM, 1 μ Ci/ml), [3 H]DHEAS (17 nM, 1 μ Ci/ml), [3 H]progesterone (20 nM, 1 μ Ci/ml), and [3 H]testosterone (20 nM, 1 μ Ci/ml) into OATP2B1-transfected and -nontransfected MDCKII cells. Cells were incubated for 15 min with the respective substance ($n = 3$).

	MDCKII Mean \pm S.D.	OATP2B1 Mean \pm S.D.	OATP2B1-Specific
	<i>fmol/min/mg</i>		
E3S	11.1 \pm 1.1	301 \pm 33*	290
DHEAS	11.4 \pm 1.9	36.3 \pm 3.5*	24.9
Progesterone	488 \pm 20	354 \pm 44*	-135
Testosterone	67.3 \pm 5.4	56.1 \pm 9.9	-11.2

* Significant vs. MDCKII for $P < 0.05$.

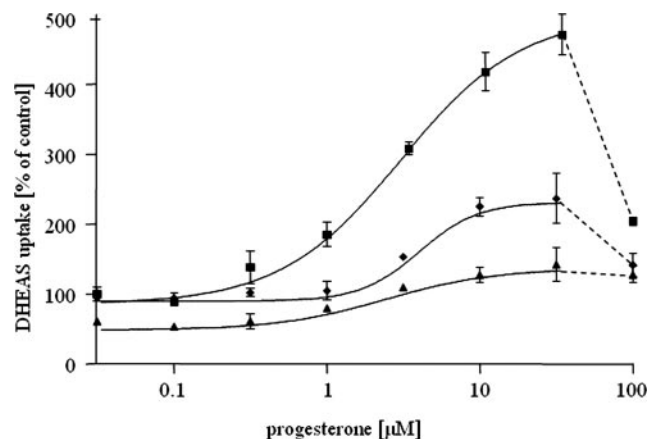


Fig. 2. Interaction of progesterone and mifepristone on DHEAS uptake into OATP2B1-overexpressing cells. Cells were incubated with [3 H]DHEAS (1 μ Ci/ml; 1 μ M) and progesterone concentrations ranging from 0 to 100 μ M for 10 min at 37°C in the absence (■) or presence of mifepristone [1 μ M (▲) or 10 μ M (◆), respectively] (mean \pm S.D.; $n = 3$ for a representative experiment).

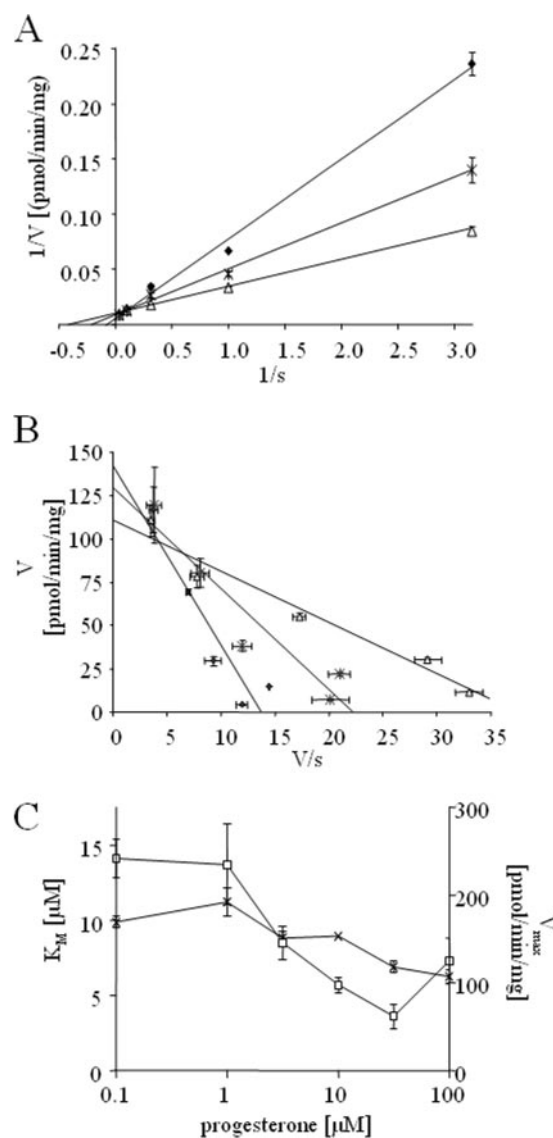


Fig. 3. Kinetic analyses of progesterone on E3S uptake. Uptake of [3 H]E3S into OATP2B1-expressing cells was measured for 5 min at E3S concentrations ranging from 0.1 to 31.6 μ M in the absence or presence of progesterone concentrations ranging from 1 to 100 μ M. A, Lineweaver-Burk plot of the transport rates of the control (◆) or 3.16 μ M (*) and 31.6 μ M (▽) progesterone incubations. B, Eadie-Hofstee plot of the same data. C, K_m (□) and V_{max} (×) values of E3S uptake plotted versus progesterone concentration. Values were calculated from the direct linear plots using GraphPad software (data represent mean \pm S.D., $n = 3$).

one concentration, 5–35 nM in women, depending on the menstrual cycle (Claydon et al., 2006)]; however, serum concentrations might not reflect local conditions. This assumption is underlined by the observation of very high concentrations in the cord blood (mean concentration: 750 nM, ranging from 240 to 1670 nM) (Baik et al., 2005) and the presence of elevated progesterone levels in nipple aspiration fluid of human breast (approximately 300 nM) (Khan et al., 2005).

Although direct transport or inhibition by steroids or its metabolites have already been described for some uptake transport proteins of the OATP family, as well as ABC-efflux pumps such as P-glycoprotein (P-gp) or breast cancer resistance protein (Frohlich et al., 2004; Pavék et al., 2005; König et al., 2006), observations of stimulatory steroid effects on uptake transport processes are rather rare. Pizzagalli et al. (2003) described stimulatory effects of prostaglandin A1 on E3S uptake mediated by OATP2B1. It is also notable that the observed effects of

progesterone and mifepristone on OATP2B1-mediated uptake of E3S and DHEAS are similar to their influence on the activity of the ABC transporter ABCB1 (P-glycoprotein, P-gp) (Fardel et al., 1996; Shapiro et al., 1999).

It is noteworthy that neither testosterone nor progesterone was transported by OATP2B1 for themselves (Table 3). Therefore, we hypothesized that testosterone blocks the E3S or DHEAS transport in a noncompetitive way. For progesterone, our observation excluded the possibility that the induction of E3S and DHEAS uptake is due to a progesterone-E3S- (DHEAS-) symport, which is underlined by the finding that no reciprocal stimulatory action of E3S or DHEAS on progesterone uptake was seen.

Further kinetic studies concerning the stimulatory effect of progesterone on E3S uptake led to the observation that progesterone increases the affinity of OATP2B1 toward E3S significantly (Fig. 3). Whether this effect is based on a direct modulatory interaction of progesterone with the substrate binding site of OATP2B1 or is due to an allosteric progesterone binding site as described by Shapiro et al. (1999) for the interaction of progesterone and P-gp remains to be elucidated. The inhibitory action of high progesterone concentrations suggests the presence of two binding sites: one high-affinity allosteric binding site, which is responsible for the stimulatory effect and the substrate binding site, or a further allosteric binding site, toward which progesterone has lower affinity and an inhibitory effect.

Moreover, we were able to show that subinhibitory concentrations of the gestagene antagonist mifepristone (approximately 1 μ M) were able to decrease the effect of progesterone on DHEAS uptake without altering the stimulatory EC_{50} concentrations of progesterone significantly, leading to the conclusion that mifepristone and progesterone are interacting in a noncompetitive way with OATP2B1 (Fig. 3). If mifepristone is thereby interacting directly with the DHEAS transport or is modulating a potential allosteric progesterone binding site remains to be elucidated. However, we assume that mifepristone has a higher affinity toward a potential allosteric progesterone binding site than progesterone. This assumption would explain our findings using low mifepristone concentrations and is supported by the fact that mifepristone acts in a similar way at the intracellular progesterone receptor.

Whereas DHEAS and E3S are already characterized as OATP2B1 substrates, pregnenolone sulfate, which represents a precursor molecule for gestagene synthesis (Chibbar and Mitchell, 1990), is not described as an OATP2B1-substrate. However, our group and others demonstrated a strong inhibitory effect on E3S and DHEAS uptake (St-Pierre et al., 2002). Moreover, in contrast to a previous study (Pizzagalli et al., 2003), we observed a small but significant OATP2B1-dependent pregnenolone sulfate transport. This transport was enhanced by progesterone with an EC_{50} concentration for progesterone comparable with the above-mentioned values.

The presented findings are especially of interest with regard to the OATP2B1 expression in hormonally regulated tissues such as placenta or mammary gland (St-Pierre et al., 2002; Pizzagalli et al., 2003). The placenta, representing the main source of estrogens and progesterone during pregnancy, is unable to convert cholesterol into estrogen because of a lack of CYP17 and is therefore highly dependent on uptake of C-19 precursor steroids such as DHEAS (Voutilainen and Miller, 1986). On the other hand, the placenta, or in more

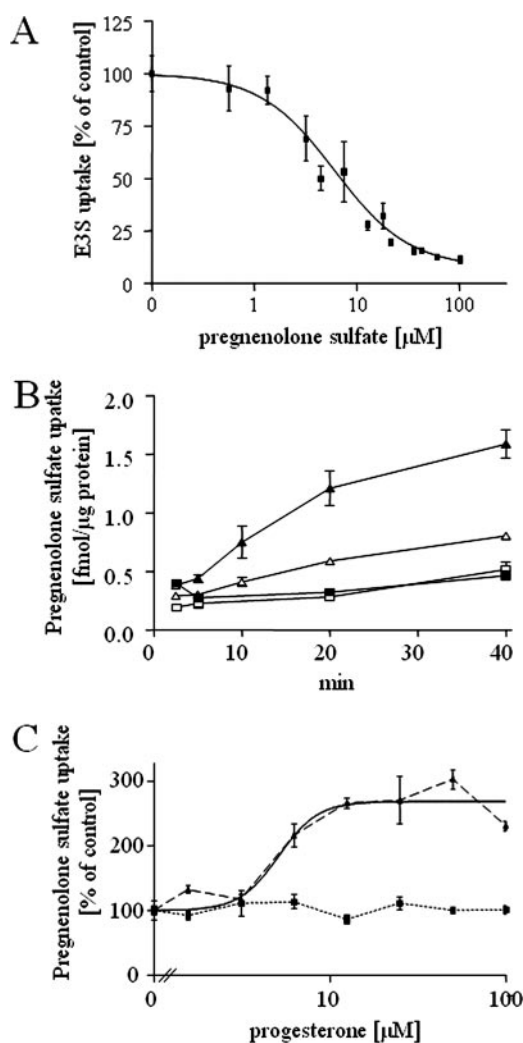


Fig. 4. Interaction of pregnenolone sulfate and OATP2B1. **A**, determination of EC_{50} value of pregnenolone sulfate on OATP2B1-mediated E3S uptake. Cells were incubated for 5 min with [3 H]E3S (1 μ Ci/ml; 1 μ M) in the presence of increasing concentrations pregnenolone sulfate. Uptake is shown as % of DMSO control (mean \pm S.D.; $n = 3$; data were obtained from three independent experiments). **B**, time course of [3 H]pregnenolone sulfate uptake (0.5 μ Ci/ml; 1 μ M) into OATP2B1-transfected MDCKII (▲) and control cells (■) in the presence (closed symbol) or absence (open symbol) of 10 μ M progesterone. **C**, uptake of [3 H]pregnenolone sulfate (0.5 μ Ci/ml; 1 μ M) into transfected (▲) and nontransfected (■) cells in the presence of different progesterone concentrations (mean \pm S.D., $n = 3$).

detail the syncytiotrophoblast, produces high levels of progesterone, which increases with the gestation age (Strauss et al., 1996; Kallen, 2004). In this context, the placental progesterone might be an important factor in regulation of DHEAS and E3S uptake. Moreover, our results also indicate a progesterone-enhanced uptake of pregnenolone sulfate via OATP2B1. This pathway represents a possible mechanism by which progesterone mediates the uptake of its own precursor molecules into the syncytiotrophoblast.

With respect to the mammary gland, circulating hormones (such as estrone-3-sulfate) and local biosynthesis within epithelial cells and stromal fibroblasts from precursors such as DHEAS and androstendione serve as estrogen-supplying mechanisms (Labrie et al., 2001). The latter mechanism becomes increasingly important during aging because estrogen synthesis by the ovaries ceases completely during menopause (Labrie et al., 2003). Similar to the placenta, the mammary gland is therefore also dependent on DHEAS and E3S uptake, and again OATP2B1 has been identified as the major uptake carrier. In contrast to the placenta, where the protein is localized to the basal membrane of the syncytiotrophoblast, here the localization is restricted to the myoepithelium, where it might play a role in the supply of desulfated hormones to the adjacent ductal epithelial cells (St-Pierre et al., 2002; Pizzagalli et al., 2003). Besides the general importance of OATP2B1 for the steroid metabolism in mammary gland, the stimulatory effect of progesterone on this transporter can be of relevance during pregnancy, when epithelial cells proliferate and milk-filled alveolar lobules develop in the mammary gland (Soyal et al., 2002; Anderson and Clarke, 2004).

Taken together, our data demonstrate strong interactions of OATP2B1-mediated uptake of E3S and DHEAS and steroid hormones. In the case of testosterone, transport of both substances was inhibited, whereas progesterone stimulated E3S and DHEAS uptake. These findings are of potential relevance for uptake regulation of sulfated steroid metabolites to sex steroid-responsive tissues such as liver, mammary gland, and placenta.

Acknowledgments

We thank Tina Brüggmann, Kerstin Böttcher, and Bärbel Uecker (Department of Pharmacology, Greifswald) for support with the experiments and for excellent technical assistance.

References

- Anderson E and Clarke RB (2004) Steroid receptors and cell cycle in normal mammary epithelium. *J Mammary Gland Biol Neoplasia* **9**:3–13.
- Baik I, Devito WJ, Ballen K, Becker PS, Okulicz W, Liu Q, Delpapa E, Lagiou P, Sturgeon S, Trichopoulos D, et al. (2005) Association of fetal hormone levels with stem cell potential: evidence for early life roots of human cancer. *Cancer Res* **65**:358–363.
- Baulieu EE (1996) Dehydroepiandrosterone (DHEA): a fountain of youth? *J Clin Endocrinol Metab* **81**:3147–3151.
- Baulieu EE and Robel P (1998) Dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEAS) as neuroactive neurosteroids. *Proc Natl Acad Sci USA* **95**:4089–4091.
- Chibbar R and Mitchell BF (1990) Steroid sulfohydrolase in human chorion and decidua: studies using pregnenolone sulfate and dehydroepiandrosterone sulfate as substrate. *J Clin Endocrinol Metab* **70**:1693–1701.
- Claydon VE, Younis NR, and Hainsworth R (2006) Phase of the menstrual cycle does not affect orthostatic tolerance in healthy women. *Clin Auton Res* **16**:98–104.
- Cui Y, König J, Leier J, Buchholz U, and Keppler D (2001) Hepatic uptake of bilirubin and its conjugates by the human organic anion transporter SLC21A6. *J Biol Chem* **276**:9626–9630.
- Fardel O, Courtois A, Drenou B, Lamy T, Lecœur V, le Prise PY, and Fauchet R (1996) Inhibition of P-glycoprotein activity in human leukemic cells by mifepristone. *Anticancer Drugs* **7**:671–677.
- Frohlich M, Albermann N, Sauer A, Walter-Sack I, Haefeli WE, and Weiss J (2004) In vitro and ex vivo evidence for modulation of P-glycoprotein activity by progestins. *Biochem Pharmacol* **68**:2409–2416.
- Grube M, Schwabedissen HM, Draber K, Prager D, Moritz KU, Linnemann K, Fusch

- C, Jedlitschky G, and Kroemer HK (2005) Expression, localization, and function of the carnitine transporter octn2 (slc22a5) in human placenta. *Drug Metab Dispos* **33**:31–37.
- Hagenbuch B and Meier PJ (2003) The superfamily of organic anion transporting polypeptides. *Biochim Biophys Acta* **1609**:1–18.
- Hagenbuch B and Meier PJ (2004) Organic anion transporting polypeptides of the OATP/SLC21 family: phylogenetic classification as OATP/SLCO superfamily, new nomenclature and molecular/functional properties. *Pflug Arch Eur J Physiol* **447**:653–665.
- Kallen CB (2004) Steroid hormone synthesis in pregnancy. *Obstet Gynecol Clin North Am* **31**:795–816.
- Khan SA, Bhandare D, and Chatterton RT Jr (2005) The local hormonal environment and related biomarkers in the normal breast. *Endocr Relat Cancer* **12**:497–510.
- Kobayashi D, Nozawa T, Imai K, Nezu J, Tsuji A, and Tamai I (2003) Involvement of human organic anion transporting polypeptide OATP-B (SLC21A9) in pH-dependent transport across intestinal apical membrane. *J Pharmacol Exp Ther* **306**:703–708.
- König J, Cui Y, Nies AT, and Keppler D (2000a) A novel human organic anion transporting polypeptide localized to the basolateral hepatocyte membrane. *Am J Physiol* **278**:G156–G164.
- König J, Cui Y, Nies AT, and Keppler D (2000b) Localization and genomic organization of a new hepatocellular organic anion transporting polypeptide. *J Biol Chem* **275**:23161–23168.
- König J, Seithel A, Gradhand U, and Fromm MF (2006) Pharmacogenomics of human OATP transporters. *Naunyn-Schmiedeberg's Arch Pharmacol* **372**:432–443.
- Kullak-Ublick GA, Fisch T, Oswald M, Hagenbuch B, Meier PJ, Beuers U, and Paumgartner G (1998) Dehydroepiandrosterone sulfate (DHEAS): identification of a carrier protein in human liver and brain. *FEBS Lett* **424**:173–176.
- Kullak-Ublick GA, Ismail MG, Stieger B, Landmann L, Huber R, Pizzagalli F, Fattinger K, Meier PJ, and Hagenbuch B (2001) Organic anion-transporting polypeptide B (OATP-B) and its functional comparison with three other OATPs of human liver. *Gastroenterology* **120**:525–533.
- Labrie F, Luu-The V, Labrie C, Belanger A, Simard J, Lin SX, and Pelletier G (2003) Endocrine and intracrine sources of androgens in women: inhibition of breast cancer and other roles of androgens and their precursor dehydroepiandrosterone. *Endocr Rev* **24**:152–182.
- Labrie F, Luu-The V, Labrie C, and Simard J (2001) DHEA and its transformation into androgens and estrogens in peripheral target tissues: intracrinology. *Front Neuroendocrinol* **22**:185–212.
- Miki Y, Nakata T, Suzuki T, Darnel AD, Moriya T, Kaneko C, Hidaka K, Shiotsu Y, Kusaka H, and Sasano H (2002) Systemic distribution of steroid sulfatase and estrogen sulfotransferase in human adult and fetal tissues. *J Clin Endocrinol Metab* **87**:5760–5768.
- Pavek P, Merino G, Wagenaar E, Bolscher E, Novotna M, Jonker JW, and Schinkel AH (2005) Human breast cancer resistance protein: interactions with steroid drugs, hormones, the dietary carcinogen 2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine, and transport of cimetidine. *J Pharmacol Exp Ther* **312**:144–152.
- Pizzagalli F, Varga Z, Huber RD, Folkers G, Meier PJ, and St-Pierre MV (2003) Identification of steroid sulfate transport processes in the human mammary gland. *J Clin Endocrinol Metab* **88**:3902–3912.
- Santner SJ, Leszczynski D, Wright C, Manni A, Feil PD, and Santen RJ (1986) Estrone sulfate: a potential source of estradiol in human breast cancer tissues. *Breast Cancer Res Treat* **7**:35–44.
- Sarkar NN (2002) Mifepristone: bioavailability, pharmacokinetics and use-effectiveness. *Eur J Obstet Gynecol Reprod Biol* **101**:113–120.
- Satoh H, Yamashita F, Tsujimoto M, Murakami H, Koyabu N, Ohtani H, and Sawada Y (2005) Citrus juices inhibit the function of human organic anion-transporting polypeptide OATP-B. *Drug Metab Dispos* **33**:518–523.
- Shapiro AB, Fox K, Lam P, and Ling V (1999) Stimulation of P-glycoprotein-mediated drug transport by prazosin and progesterone. Evidence for a third drug-binding site. *Eur J Biochem* **259**:841–850.
- Skalba P, Wojtowicz M, and Sikora J (2003) Androgen and SHBG serum concentrations in late post-menopause women. *Med Sci Monit* **9**:CR152–CR156.
- Soyal S, Ismail PM, Li J, Mulac-Jericevic B, Conneely OM, and Lydon JP (2002) Progesterone's role in mammary gland development and tumorigenesis as disclosed by experimental mouse genetics. *Breast Cancer Res* **4**:191–196.
- St-Pierre MV, Hagenbuch B, Ugele B, Meier PJ, and Stallmach T (2002) Characterization of an organic anion-transporting polypeptide (OATP-B) in human placenta. *J Clin Endocrinol Metab* **87**:1856–1863.
- Strauss JF 3rd, Martinez F, and Kiriakidou M (1996) Placental steroid hormone synthesis: unique features and unanswered questions. *Biol Reprod* **54**:303–311.
- Tamai I, Nezu J, Uchino H, Sai Y, Oku A, Shimane M, and Tsuji A (2000) Molecular identification and characterization of novel members of the human organic anion transporter (OATP) family. *Biochem Biophys Res Commun* **273**:251–260.
- Tamai I, Nozawa T, Koshida M, Nezu J, Sai Y, and Tsuji A (2001) Functional characterization of human organic anion transporting polypeptide B (OATP-B) in comparison with liver-specific OATP-C. *Pharm Res (NY)* **18**:1262–1269.
- Ugele B, St-Pierre MV, Pihusch M, Bahn A, and Hantschmann P (2003) Characterization and identification of steroid sulfate transporters of human placenta. *Am J Physiol* **284**:E390–E398.
- Voutilainen R and Miller WL (1986) Developmental expression of genes for the steroidogenic enzymes P450scc (20,22-desmolase), P450c17 (17 α -hydroxylase/17,20-lyase), and P450c21 (21-hydroxylase) in the human fetus. *J Clin Endocrinol Metab* **63**:1145–1150.

Address correspondence to: Dr. Heyo K. Kroemer, Department of Pharmacology, Friedrich-Loeffler-Str. 23d, 17487 Greifswald, Germany. E-mail: kroemer@uni-greifswald.de