Pharmaceutical Research, Vol. 22, No. 10, October 2005 (© 2005)
DOI: 10.1007/s11095-005-7096-0

Research Paper

Suppression of Cell Proliferation by Inhibition of Estrone-3-Sulfate Transporter in Estrogen-Dependent Breast Cancer Cells

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Received February 1, 2005; accepted June 28, 2005

Purpose. The aim of the study is to suppress the progress of estrogen-dependent breast cancer by inhibiting the membrane transporter, which mediates the internalization of estrone-3-sulfate as estrogen precursor in the cancer cells.
Methods. The uptake of estrone-3-sulfate by estrogen-dependent breast cancer MCF-7 cells was measured, and inhibitory study using various organic anions on estrone-3-sulfate uptake by MCF-7 cells was conducted. The effects of the inhibitor on the transcription of reporter gene and cell proliferation induced by estrone-3-sulfate were examined.
Results. The uptake of estrone-3-sulfate by MCF-7 cells was saturable with K_m value of 2.32 μM. The uptake was Na^+-independent and was inhibited by several anionic compounds such as bromosulfophthalein. Bromosulfophthalein also significantly inhibited the transcription of reporter gene via estrogen response element and cell proliferation induced by estrone-3-sulfate. However, the transcriptional activation or cell proliferation induced by estrone was not inhibited by bromosulfophthalein. Reverse transcription–polymerase chain reaction analysis revealed the expression of mRNA of organic anion transporting polypeptide (OATP)-D and OATP-E as possible candidates to transport estrone-3-sulfate.
Conclusions. The uptake of estrone-3-sulfate is mediated by Na^+-independent transporter(s). Inhibitor of estrone-3-sulfate transporter suppressed the transcription and cell proliferation induced by estrone-3-sulfate in MCF-7 cells. The results provide the basis of a novel strategy for breast cancer treatment by focusing on the transporter responsible for the uptake of estrone-3-sulfate.

KEY WORDS: breast cancer; bromosulfophthalein; estrone-3-sulfate; estrogen-dependent cell proliferation; MCF-7; transporter.

INTRODUCTION

Breast cancer is one of the major causes of death in women by cancer, especially postmenopausal women. Estrogen is an important risk factor for the progression of breast tumors because two thirds of breast cancer cells are estrogen-sensitive, i.e., their growth is regulated by estrogens (1). Estrone-3-sulfate is one of the most important forms of circulating estrogens, because its plasma concentration is about five to ten times higher than that of unconjugated estrogens, and its half-life is longer than that of estradiol in postmenopausal woman (2–4). Although estrone-3-sulfate itself is biologically inactive at the estrogen receptor (ER), it is converted to active estrogen, estradiol, by estrogen sulfatase and 17β-hydroxysteroid dehydrogenase type 1 in the cells (5–8). Moreover, the expression level of sulfatase mRNA in breast cancers is about 3-fold higher than that in normal tissues, suggesting efficient conversion of estrone-3-sulfate to active estrogen in breast cancer cells (9). However, estrone-3-sulfate is highly hydrophilic, so that it cannot readily cross the plasma membrane by diffusion, whereas unconjugated estrogens, estrone and estradiol, are lipophilic and can enter cells via simple diffusion (10,11). Accordingly, it is expected that a transporter for estrone-3-sulfate is expressed in hormone-dependent breast cancer cells. Indeed, we previously reported that the uptake of estrone-3-sulfate across the plasma membrane in estrogen-dependent T-47D breast cancer cells was mediated by a specific transport mechanism (12). Moreover, the uptake of estrone-3-sulfate by T-47D cells was sodium-independent and strongly inhibited by an organic anion, bromosulfophthalein (BSP). Accordingly, we hypothesized that this transporter could be a novel target for endocrine therapy of estrogen-dependent breast cancer.

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ABBREVIATIONS: BSP, bromosulfophthalein; DCC, dextran-coated charcoal; DMSO, dimethyl sulfoxide; ER, estrogen receptor; ERE, estrogen response element; FCS, fetal calf serum; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; OAT, organic anion transporter; OATP, organic anion transporting polypeptide; SEAP, secreted alkaline phosphatase; SLC, solute carrier family.

Many membrane transporters have been cloned and functionally analyzed. Furthermore, considerable effort has been made to examine their clinical potential, e.g., as targets for chemotherapy. Several members of the solute carrier family (SLC), as well as ABC transporters such as P-glycoprotein and multidrug resistance associated proteins (MRPs), are expressed in various tumor cells and are involved in drug sensitivity (13,14). Na^+-taurocholate-cotransporting polypeptide (SLC10A1) and organic anion-transporting polypeptide OATP1B3 (formerly termed OATP8, SLC21A8) are expressed in hepatocellular and gastrointestinal carcinomas, respectively (15,16). Moreover, although the responsible molecule has not been identified, transport activity for di-and tripeptides and peptide-mimetic drugs has been observed in various cancer cell lines (17,18). Therefore, these transporters may be applicable to deliver chemotherapeutic agents to target cancer cells. In contrast, L-type amino acid transporter 1 (LAT1, SLC7A5) is expressed in leukemia cell lines and is responsible for the transport of large neutral amino acids, including several essential amino acids (19). The inhibition of such a transporter should deplete the supply of nutrients, leading to suppression of cancer progression. As mentioned above, we found that estrone-3-sulfate is taken up by a specific transporter in estrogen-dependent breast cancer cells (12). Therefore, inhibition of this transporter should decrease the accumulation of estrone-3-sulfate and reduce the effective estrogenic activity, i.e., suppress the progression of breast cancer.

In the present study, to establish whether the estrone-3-sulfate transporter would be a good candidate as a novel target for treatment of breast cancer, we examined the uptake of estrone-3-sulfate and the effect of a transporter inhibitor on proliferation and estrogenic activity, using the estrogen-dependent breast cancer cell line MCF-7.

MATERIALS AND METHODS

Materials

[^{3}H]Estrone-3-sulfate, ammonium salt (1,702.0 GBq/mmol), was purchased from Perkin-Elmer Life Science Products, Inc. (Boston, MA, USA). MCF-7 cells were purchased from American Type Culture Collection (ATCC, Rockville, MA, USA). Fetal calf serum (FCS) was obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). Mercury™ pathway profiling SEAP system 2 was purchased from BD Bioscience (Bedford, MA, USA). All other reagents were purchased from Sigma Chemicals (St. Louis, MO, USA) and Wako Pure Chemical Industries (Osaka, Japan).

Transport Experiments

Transport experiments were performed as described previously (20). MCF-7 cells were routinely grown in Dulbecco's modified Eagle's medium containing 10% FCS in a humidified incubator at 37°C under 5% CO_2. After cultivation of MCF-7 cells in 15-cm dishes, the cells were harvested and suspended in the transport medium containing 125 mM NaCl, 4.8 mM KCl, 5.6 mM D-glucose, 1.2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, and 25 mM HEPES, adjusted to pH 7.4. The cell suspension and a solution containing [^{3}H]estrone-3-sulfate in the transport medium were separately incubated at 37°C for 20 min, then transport was initiated by mixing them. At appropriate times, 150-μL aliquots of the mixture were withdrawn, and the cells were separated from the transport medium by centrifugal filtration through a layer of a mixture of silicone oil (SH550, Toray Dow Corning Co., Tokyo, Japan) and liquid paraffin (Wako Pure Chemical Industries) with a density of 1.03. Each cell pellet was solubilized in 3 N KOH and neutralized with HCl. The associated radioactivity was measured by means of a liquid scintillation counter using Clearsol-1 as a liquid scintillation fluid (Nacalai tesque, Kyoto, Japan).

Reporter Gene Assay

MCF-7 cells were plated at 1×10^5 cells/well in 24-well plates (BD Bioscience) the day before transfection. For detection of secreted alkaline phosphatase (SEAP) activity induced via estrogen response element (ERE), pSEAP2 control or pERE-TA-SEAP vector (BD Bioscience) was transiently transfected using Lipofectamine™ 2000 (Invitrogen Life Technologies) according to the manufacturer's instructions. At 24 h after transfection, the medium was replaced with phenol red-free Dulbecco's modified Eagle's medium containing 2.5% dextran-coated charcoal (DCC)-treated FCS and estrone-3-sulfate, with or without inhibitor. Supernatants were harvested at 8 h for detection of SEAP activity. The SEAP activity was measured using a SEAP assay kit (BD Bioscience) with a fluorescence multiwell plate reader (CytoFluor™II, PerSeptive Biosystems, Inc., Framingham, MA, USA) according to the manufacturer's instructions.

Cell Proliferation Assay

MCF-7 cells were plated at 8,000 cells/well in 96-well plates (Iwaki, Osaka, Japan) in phenol red-free Dulbecco's modified Eagle's medium containing 2.5% DCC-treated FCS. At 24 h after seeding, estrone or estrone-3-sulfate was added at graded concentrations from stock solutions in water for estrone-3-sulfate or dimethyl sulfoxide (DMSO 0.1%) for estrone. The negative control included solvent alone. At 72 h after seeding, the cells were treated with trypsin, and cell numbers were counted.

Reverse Transcription–Polymerase Chain Reaction Method

Expression of OATP and organic anion transporter (OAT) in MCF-7 cells was examined by the reverse transcription–polymerase chain reaction (RT-PCR) method as described previously (12). Single-stranded cDNA was constructed using an oligo(dT) primer (Invitrogen Corp.). The specific primers designed in previous report were used for PCR. The reaction was performed 94°C for 2 min, 35 cycles of 94°C for 30 s, 58°C (OATPs) or 56°C (OATs) for 30 s, 72°C for 30 s, and final elongation at 72°C for 10 min in the presence of deoxynucleotides and *Tag* polymerase (Takara Bio Inc., Shiga, Japan). Polymerase chain reaction products

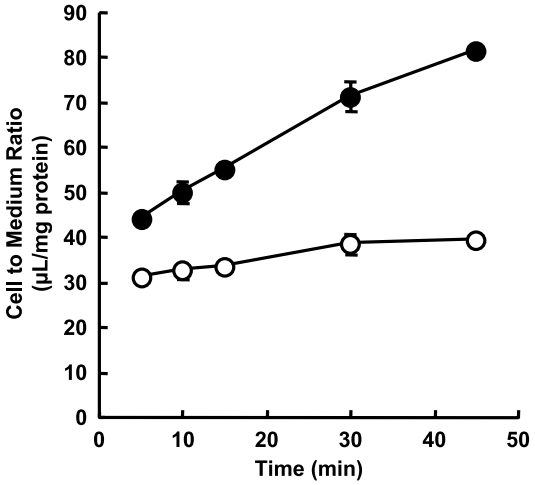

Fig. 1. Time course of [^{3}H]estrone-3-sulfate uptake by MCF-7 cells. Cultured MCF-7 cells were incubated at 37°C over 45 min in medium containing [^{3}H]estrone-3-sulfate (10 nM) with (opened circles) or without (closed circles) 1 mM unlabeled estrone-3-sulfate. Each value represents the mean ± SEM (n = 4). When the error bars are not shown, they are smaller than the symbols.

were analyzed by means of 2% agarose gel (w/v) electrophoresis, and the gel was stained with ethidium bromide to visualize bands.

Analytical Methods

Cellular protein content was determined according to the method of Bradford (21) by using a BioRad protein assay kit (Hercules, CA, USA) with bovine serum albumin as the standard. Uptake rate of estrone-3-sulfate by MCF-7 cells was evaluated in terms of the uptake at 30 min after subtraction of the uptake in the presence of an excess of unlabeled estrone-3-sulfate (1 mM). To estimate the kinetic parameters of saturable transport, the uptake rate (v) was fitted to the following equations by means of nonlinear least-squares regression analysis using KaleidaGraph™ (Synergy, PA, USA).

$$v = V_{max} * s/(K_m + s)$$

where v and s are the uptake rate and concentration of substrate, respectively, and K_m and V_{max} represent the half-saturation concentration (Michaelis constant) and the maximum uptake rate, respectively.

All data were expressed as means ± SEM, and statistical analysis was performed by the use of Student's t test with $p < 0.05$ as the criterion of significance. Cell-to-medium ratio was obtained by dividing the cellular uptake amount by the concentration of test compound in the uptake medium.

RESULTS

Characteristics of Estrone-3-Sulfate Uptake by MCF-7 Cells

Figure 1 shows the time course of the uptake of [^{3}H]estrone-3-sulfate (10 nM) by MCF-7 cells in the presence or absence of unlabeled 1 mM estrone-3-sulfate. The uptake of [^{3}H]estrone-3-sulfate was increased over 45 min. In the presence of unlabeled 1 mM estrone-3-sulfate, no significant increase in the uptake of [^{3}H]estrone-3-sulfate was observed over 45 min, suggesting that estrone-3-sulfate was unlikely to be taken up into MCF-7 cells by simple diffusion. Based on these results, the apparent uptake values were analyzed after subtraction of the nonsaturable uptake obained in the presence of unlabeled estrone-3-sulfate to correct

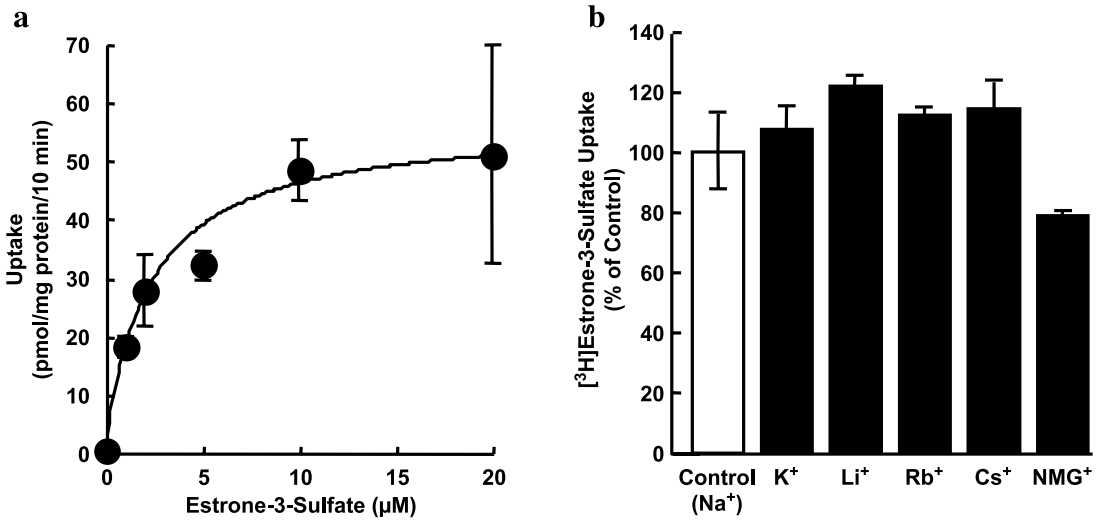

Fig. 2. Concentration dependence and effect of extracellular cations on uptake of estrone-3-sulfate by MCF-7 cells. (a) Uptake of estrone-3-sulfate at various concentrations ranging from 10 nM to 30 μM was measured at 37°C for 10 min. The saturable uptake was calculated by subtraction of the uptake in the presence of excess of unlabeled estrone-3-sulfate (1 mM) and used for the evaluation of kinetic constants by nonlinear least-squares analysis. (b) Uptake of [^{3}H]estrone-3-sulfate (10 nM) for 30 min was measured in the presence or absence of extracellular Na^+. Na^+ was replaced with K^+, Li^+, Rb^+, Cs^+, or *N*-methylglucamine$^+$ (NMG^+). Each value represents the mean ± SEM (n = 4). When the error bars are not shown, they are smaller than the symbols.

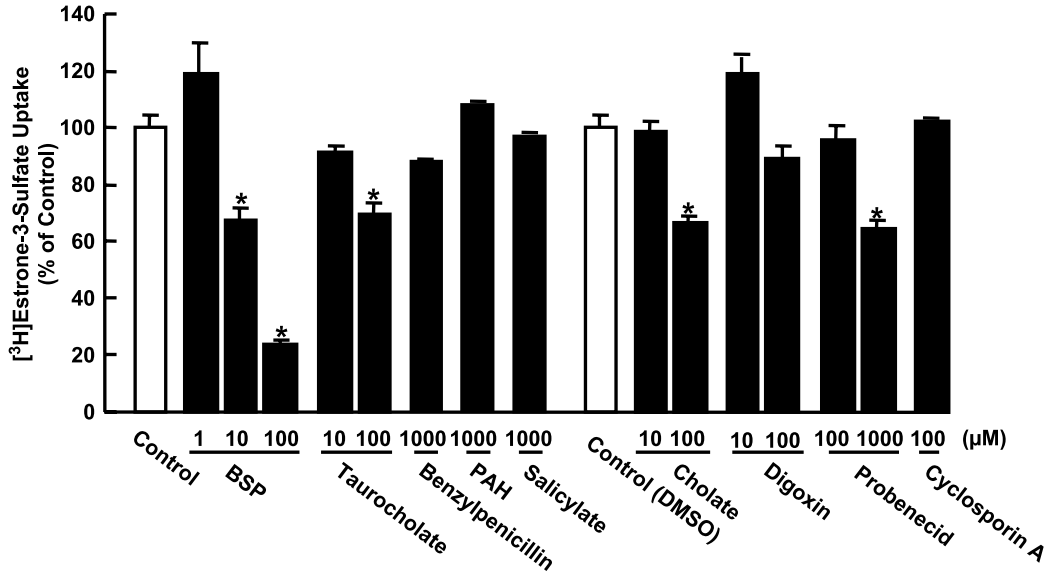

Fig. 3. Inhibitory effect of various compounds on [^{3}H]estrone-3-sulfate uptake by MCF-7 cells. Cultured MCF-7 cells were incubated at 37°C for 30 min in medium containing [^{3}H]estrone-3-sulfate (10 nM) with (closed column) or without (open column, control) an inhibitor at the indicated final concentration. Transporter-mediated uptake was calculated by subtracting the uptake in the presence of excess estrone-3-sulfate (1 mM) from the total uptake. Uptake was expressed as percent of the control uptake. Each value represents the mean ± SEM (n = 4). *$p < 0.05$, compared with the control (Student's t test).

for adsorption on the cell surface and uptake by simple diffusion, and the uptake of estrone-3-sulfate at 10 min by MCF-7 cells was kinetically analyzed in the following studies. The uptake of estrone-3-sulfate was saturable with K_m and V_{max} values (mean ± SE) of 2.32 ± 1.20 μM and 56.3 ± 8.6 pmol/mg protein/10 min, respectively (Fig. 2a). Furthermore, the effect of replacement of Na^+ with various cations on estrone-3-sulfate uptake was examined (Fig. 2b). When Na^+ was replaced with K^+, Li^+, Rb^+, Cs^+, or *N*-methylglucamine$^+$, the uptake of [^{3}H]estrone-3-sulfate was comparable with that in the presence of Na^+, suggesting the involvement of an Na^+-independent transport mechanism.

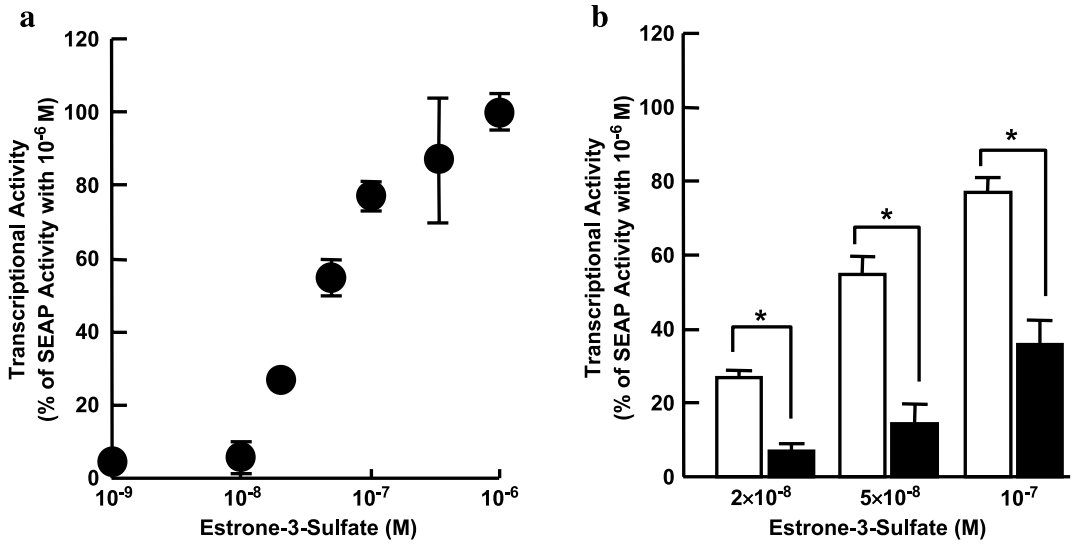

Fig. 4. Dose dependence and effect of bromosulfophthalein (BSP) on secreted alkaline phosphatase (SEAP) activity by addition of estrone-3-sulfate in MCF-7 cells. Cultured MCF-7 cells were transfected with pERE-TA-SEAP vector. (a) SEAP activity at various concentration of estrone-3-sulfate in the range from 1 nM to 1 μM was measured. (b) The inhibitory effect of BSP on transcriptional activity induced by estrone-3-sulfate was examined at 20, 50, or 100 nM of estrone-3-sulfate. The open and closed columns represent the results obtained in the absence and presence of BSP (100 μM), respectively. Each point represents a percentage of SEAP activity obtained at 10^{-6} M of estrone-3-sulfate in the absence of BSP and is expressed as mean ± SEM (n = 4). *$p < 0.05$, compared with the control (Student's t test).

Inhibitory Effects of Various Compounds on the Uptake of Estrone-3-Sulfate

To find inhibitors of estrone-3-sulfate uptake by MCF-7 cells, various compounds were tested (Fig. 3). Among them, BSP showed the strongest inhibition, whereas taurocholate, probenecid, and cholate inhibited the uptake less potently, but still with statistical significance. Salicylate, *p*-aminohippuric acid, benzylpenicillin, digoxin, and cyclosporin A were not inhibitory. Therefore, we considered that BSP should be a potent inhibitor of the proliferation of MCF-7 cells induced by estrone-3-sulfate.

Effect of Transporter Inhibitor on Estrone-3-Sulfate-Induced Transcriptional Activation

Estrogen dependence is mainly mediated via the binding of estrogen to ER, and the complex can mediate the transcriptional activation of target genes, including proto-oncogenes, oncogenes, and nuclear proteins, via ERE. In the present study, we examined transcriptional activation of estrogen by using pERE-TA-SEAP vector, which contains SEAP gene located downstream of an ERE. Estrone-3-sulfate is hydrophilic and requires a specific transporter to cross the cell membrane, whereas unconjugated estrogen, estrone, is hydrophobic and diffusible (10,11). Because the uptake of estrone by MCF-7 cells was not inhibited by BSP (data not shown), we used estrone as a reference compound. When pERE-TA-SEAP vector was transfected in MCF-7 cells, the SEAP activity was increased by addition of estrone-3-sulfate or estrone in a concentration-dependent manner (Figs. 4a and 5a). The SEAP activity attained a plateau with an increase of estrone-3-sulfate or estrone concentration, and the apparent EC_{50} values were about 50 nM and 0.5 pM after subtraction of the SEAP activities in the absence of estrone-3-sulfate or estrone, respectively. The SEAP activity induced by 20, 50, or 100 nM estrone-3-sulfate was significantly decreased in the presence of 100 μM BSP (Fig. 4b). In contrast, the SEAP activity induced by 0.1 or 1 pM estrone was not affected by 100 μM BSP (Fig. 5b). Accordingly, BSP effect is considered to be specific to estrone-3-sulfate.

Effect of Transport Inhibitor on Estrogen-Induced Cell Proliferation

Next, we examined directly whether BSP can inhibit estrogen-induced cell proliferation. When MCF-7 cells were treated with estrone or estrone-3-sulfate, the cell number was increased in a concentration-dependent manner (Table I). The apparent EC_{50} values were 0.4 and 60 nM for estrone and estrone-3-sulfate, respectively (based on the observed cell number minus the cell number in the absence of estrogen). The addition of BSP at 30 or 100 μM significantly reduced the cell proliferation in the presence of 10^{-7}–10^{-5} M estrone-3-sulfate, whereas BSP at 100 μM increased the cell proliferation induced by 10^{-8} M estrone-3-sulfate. In contrast, BSP had no effect on the cell proliferation induced by 10^{-10}–10^{-7} M estrone.

Expression of OAT and OATP Transporters

Finally, to identify the transporters that mediate the uptake of estrone-3-sulfate in MCF-7 cells, we investigated the expression of OATP and OAT transporters, which can potentially accept estrone-3-sulfate as substrate, by RT-PCR analysis. Figure 6 shows the signals of expression of OAT4,

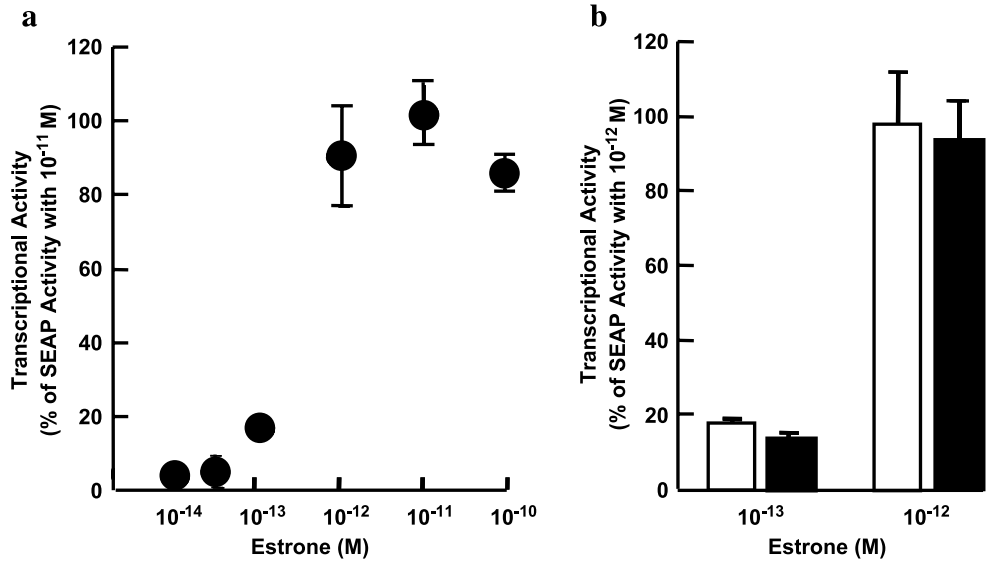

Fig. 5. Dose dependence and effect of BSP on SEAP activity by addition of estrone in MCF-7 cells. Cultured MCF-7 cells were transfected with pERE-TA-SEAP vector. (a) SEAP activity at various concentration of estrone in the range from 0.01 to 100 pM was measured. Each point represents a percentage of SEAP activity obtained at 10^{-11} M of estrone and is expressed as mean ± SEM (n = 4). (b) The inhibitory effect of BSP (100 μM) on transcriptional activity induced by estrone at 0.1 and 1 pM was examined. The open and closed columns represent the results obtained in the absence and presence of BSP (100 μM), respectively. Each point represents a percentage of SEAP activity obtained at 10^{-12} M of estrone and is expressed as mean ± SEM (n = 4).

Table I. Dose-Dependent Stimulation of Estrogen-Induced Cell Proliferation and the Inhibitory Effect of BSP

	Percentage of cell number with 10^{-7} M estrone					
Estrone	10^{-12} M	10^{-11} M	10^{-10} M	10^{-9} M	10^{-8} M	10^{-7} M
Control	10.9 ± 10.1	26.2 ± 11.1	31.6 ± 7.7	65.1 ± 11.7	110.5 ± 12.9	100 ± 13.4
+30 μM BSP	–	–	43.3 ± 9.2	64.0 ± 8.3	101.5 ± 8.5	105.1 ± 21.1
+100 μM BSP	–	–	59.6 ± 14.4	73.1 ± 17.0	91.8 ± 9.1	88.5 ± 8.6
	Percentage of cell number with 10^{-5} M estrone-3-sulfate					
Estrone-3-sulfate	10^{-10} M	10^{-9} M	10^{-8} M	10^{-7} M	10^{-6} M	10^{-5} M
Control	18.2 ± 4.2	25.5 ± 1.1	32.8 ± 2.5	62.1 ± 1.8	107.6 ± 6.8	100 ± 4.7
+30 μM BSP	–	–	33.7 ± 4.7	47.8 ± 1.2*	60.6 ± 2.3*	69.7 ± 1.7*
+100 μM BSP	–	–	52.1 ± 4.3*	47.5 ± 4.8*	40.3 ± 7.9*	48.2 ± 5.8*

MCF-7 cells were seeded at the density of 8,000 cells/well and cultured. Twenty-four hours after seeding, cells were exposed to estrone (10^{-12}–10^{-7} M) or estrone-3-sulfate (10^{-10}–10^{-5} M) with or without BSP at 30 and 100 μM. At 72 h after seeding, the cells were trypsinized and counted. Estrogen-induced cell proliferation was calculated after subtraction of the counts in the absence of estrogen. Cell numbers were expressed as a percentage of those obtained with estrone (10^{-7} M) and estrone-3-sulfate (10^{-5} M), respectively. Each value represents mean ± SEM (n = 4).
–, Not tested; BSP, bromosulfophthalein.
*$p < 0.05$, compared with the corresponding control (Student's t test).

OATP-A, OATP-D, and OATP-E in MCF-7 cells. However, the signals of OAT4 and OATP-A were weak compared with those of OATP-D and OATP-E. Thus, they may be candidates to mediate the uptake of estrone-3-sulfate in estrogen-dependent breast cancer cells.

DISCUSSION

Estrogen induces the progression of estrogen-dependent breast cancers, and consequently, antiestrogens have been developed for breast cancer treatment. Those drugs mainly target ER or enzymes, such as aromatase, sulfatase, and 17β-hydroxysteroid dehydrogenase, that activate estrogens to estradiol in the cells (2,22,23). We considered that the membrane transporter responsible for estrone-3-sulfate uptake would be a novel target for breast cancer treatment. Estrone-3-sulfate is a major circulating estrogen and is involved in the progression of estrogen-dependent breast cancer in postmenopausal woman (2). We previously reported that the internalization of estrone-3-sulfate is mediated by a specific transporter in estrogen-dependent T-47D cells, although the transporter was not identified at the molecular level (19). In the present study, we examined whether inhibition of the transporter leads to inhibition of estrogenic activity, including transcriptional activation of target genes and estrogen-dependent cell proliferation.

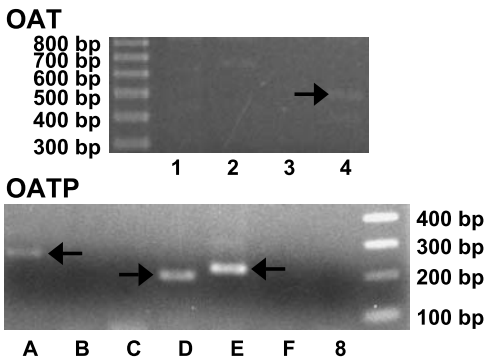

Fig. 6. Expression of organic anion transporting polypeptide (OATP) and organic anion transporters (OATs) in MCF-7 cells. Reverse transcription–polymerase chain reaction analysis was performed using mRNA obtained from MCF-7 cells. The reactions using specific primers for OATP or OAT were performed as described in Materials and Methods. The arrows show the specific bands of OAT4, OATP-A, OATP-D, and OATP-E.

First of all, we confirmed the involvement of a specific transporter, which could transport estrone-3-sulfate into MCF-7 cells. The uptake of [^{3}H]estrone-3-sulfate was saturable with a K_m value of 2.32 μM and Na^+-independent. The K_m value in MCF-7 cells was similar with that in T-47D cells (7.6 μM), suggesting the involvement of the same transporter (12). This K_m value is also consistent with the known range of K_m values of OATPs and OATs of 0.05–59 μM (24,25). Moreover, the inhibitory effects of BSP and taurocholate, which can be transported by several organic anion transporters, are also consistent with the involvement of such known transporter(s). Because molecular identification is required to underpin the proposed treatment strategy, we conducted RT-PCR analysis of OATPs and OATs (Fig. 6). We detected mRNA of OAT4, OATP-A, OATP-D, and OATP-E among tested OATs and OATPs. OATP-D and OATP-E are also present in T-47D, which is estrogen-dependent breast cancer cell line (12,26). The expression of OAT4 and OATP-A suggested that they are also potent to transport estrone-3-sulfate in MCF-7 cells (24,25). However, their signals in PCR analysis were weak at 35 cycles, and they were not detectable in T-47D cells, which have similar transport activity for estrone-3-sulfate, although further quantitative analysis may be required to compare their expression levels (12). Moreover, because the inhibitory effects of bile acids, which are typical substrates of OATP, were not so strong (Fig. 3), the contributions of OATP-D and OATP-E to the uptake of estrone-3-sulfate in MCF-7 cells remain to be established. In reporter gene assay, estrone-3-sulfate induced transcriptional activation, seen as an incre-

ment of SEAP activity (Fig. 4a), as the same as observed by addition of estrone (Fig. 5a). Because estrone-3-sulfate itself is biologically inactive at the ER, estrone-3-sulfate is presumably transported across the plasma membrane and hydrolyzed by intracellular steroid sulfatase to active estrogen, estrone or estradiol. Moreover, estrone-3-sulfate induced proliferation of MCF-7 cells in a concentration-dependent manner. The EC_{50} values of estrone-3-sulfate were comparable in reporter gene assay (50 nM) and cell proliferation assay (60 nM) and different from the K_m value of the transporter (2.32 μM). Therefore, the rate-determining step in the acquisition of estrogenic activity may not be determined only by the transport step across the cell membrane, but some other steps, such as metabolism to active estrogen, and binding to ER or ERE (6–8). Moreover, because the ER complex interacts with other proteins, including coactivators, corepressors, and integrator proteins (27), these findings are not sufficient in themselves to establish the contribution of the transport step of estrone-3-sulfate to the complex processes leading to acquisition of estrogenic activity.

We next examined the ability of BSP to inhibit estrogenic effects induced by estrone-3-sulfate. Secreted alkaline phosphatase activity was significantly inhibited by BSP at 100 μM (Fig. 4). In addition, BSP at 30 and 100 μM also inhibited the estrone-3-sulfate-induced cell proliferation in a concentration-dependent manner (Table I). In contrast, BSP had no effect on the estrone-induced estrogenic effect, including transcriptional activity (Fig. 5b) and cell proliferation (Table I). Although it was not established whether BSP reduced the estrogenic effect only by inhibition of the transport process, the absence of any effect of BSP on estrone indicates that BSP acted on a process specific to estrone-3-sulfate, i.e., transport or hydrolysis. Although we should examine whether BSP also inhibits steroid sulfatase or not, we can conclude that the transporter of estrone-3-sulfate plays an important role in the acquisition of estrogenic activity, and inhibition of the estrone-3-sulfate transporter at least partly suppresses the progression of estrogen-dependent breast cancers. On the other hand, 100 μM BSP increased cell proliferation in the presence of 10 nM estrone-3-sulfate, which is comparable to the plasma concentration in women (2). Bromosulfophthalein might itself have estrogenic activity in the presence of a low concentration of estrogen, and this would be disadvantageous for a drug candidate. Moreover, the effective concentrations of BSP (30 and 100 μM) seem high considering the usual plasma concentration. Structural modification studies to isolate the inhibitory effect of BSP on the uptake of estrone-3-sulfate are needed to develop drug candidates for breast cancer treatment.

Various enzyme and receptor inhibitors have so far been developed for breast cancer treatment, as mentioned above (2,22,23). Although these molecular targets are localized in intracellular domains, transporters are membrane proteins and face the outside of the cells. Therefore, drugs can act on the transporters without having to permeate across the cell membrane. Thus, specific antibodies against the extracellular domains or drugs that are not readily transported into the cells, because of high hydrophilicity or large molecular size, are also available as candidate anticancer drugs. Moreover, the expression of target molecules is an important criterion of usefulness in breast cancer treatment. For example, tamoxifen and trastuzumab are effective against breast cancers expressing ER and HER-2 receptor, respectively (27,28). Furthermore, it is reported that the expression pattern of ER changes with the evolution of breast cancers (2). Therefore, studies on the precise molecular identification and expression pattern of the estrone-3-sulfate transporter in breast cancer cells are now needed.

In conclusion, we have clarified that the uptake of estrone-3-sulfate in hormone-dependent MCF-7 cells is mediated by a specific transporter, and inhibition of the transporter leads to suppression of the transcriptional and cell proliferation activities of estrone-3-sulfate. This may provide the basis of a novel strategy for breast cancer treatment by focusing on the transporter responsible for the uptake of estrone-3-sulfate.

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