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# Estrogen Sulfotransferase Expression in the Human Liver: Marked Interindividual Variation and Lack of Gender Specificity

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## ABSTRACT

Estrogen sulfotransferase (EST) catalyzes the specific sulfonation of estrogen at the 3′-hydroxyl position using 3′-phosphoadenosine-5′-phosphosulfate as an activated sulfate donor. Sulfonation renders the hormone biologically inactive as well as changing its half-life within the human body. Studies in the rat and mouse have suggested that expression of EST in the liver is age- and sex-dependent, being prominent only in sexually mature young males. Although a human EST cDNA has previously been cloned, the characteristics of hepatic EST expression in human subjects remain to be defined. In this study, we have investigated and compared the expression of EST in 10 human liver samples by using an EST-specific antibody and performing enzyme activity assays. We found a marked interindividual variation (up to 25-fold) in the hepatic expression of EST. However, EST protein level in the human liver is correlated neither with gender nor with age. Interestingly, paired-group analysis revealed a statistically significant difference in the hepatic expression of EST protein and activity between alcohol users and nonusers. We conclude that, unlike what is observed in the rodent liver, EST expression in the human liver is not sex-limited. Thus hepatic EST may play a role in estrogen metabolism and homeostasis in both genders of human subjects. The marked individual variation suggests that EST gene expression is subject to sensitive control by genetic or environmental factors. The potential correlation between alcohol consumption and hepatic EST expression deserves further evaluation.

Sulfotransferases are cytosolic enzymes that catalyze the sulfonation of both xenobiotics and endogenous compounds (Falany, 1997). The best-characterized members of this family of enzymes are the PST, which are responsible for sulfoconjugating monoamine neurotransmitters (M-PST) and drugs or xenobiotic compounds such as phenols (P-PST) (Wilborn *et al.*, 1993; Ozawa *et al.*, 1995; Zhu *et al.*, 1993; Weinshilboum *et al.*, 1997). Although steroid sulfotransferase activities in the liver of animals and the human have been well recognized and studied, the identity and substrate specificity of the relevant enzymes remained poorly defined until recently. For a long time, the term *steroid sulfotransferase* was used indiscriminately, which implied the existence of a single enzyme responsible for sulfonating all compounds with a steroid nucleus structure. From recent protein purification and molecular cloning studies, we now know that two steroid sulfotransferases are expressed in the liver of humans and animals (Strott, 1997; Weinshilboum *et al.*, 1997). A HSST preferentially metabolizes 3$\beta$-hydroxysteroids such as pregnenolone and DHEA, and an EST is specific for conjugating estrogens. Pharmacokinetic studies of purified or heterologously expressed enzymes have unambiguously distinguished EST from other sulfotransferases (Moore *et al.*, 1988; Song *et al.*, 1995; Falany *et al.*, 1994, 1995). Although both PST and HSST have been reported to possess estrogen-sulfonating activity (Falany *et al.*, 1989, 1994; Hernandez *et al.*, 1992), the $K_m$ value of EST for estrogens is in the nanomolar range and is at least three orders of magnitude lower than that of PST or HSST (Falany *et al.*, 1994, 1995; Falany and Falany, 1996). Thus, under physiological conditions, one would expect EST to be the sulfotransferase most relevant in the sulfo-conjugation of estrogens *in vivo*.

Studies in the rat and mouse have shown that the hepatic expression of EST is sexually dimorphic, being prominent only in sexually mature and competent males (Demyan *et al.*, 1992; Song *et al.*, 1997). It has also been demonstrated that expression of EST in the liver is dramatically induced in the genetically obese and diabetic *db*/*db* mice (Leiter and Chapman, 1994; Song *et al.*, 1995). Although the human EST cDNA has previously been cloned (Aksoy *et al.*, 1994; Falany *et al.*, 1995), the pattern of its expression and that of its regulation in the human liver have not been characterized.

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**ABBREVIATIONS:** CHO, Chinese hamster ovary; DHEA, dehydroepiandrosterone; EST, estrogen sulfotransferase; HSST, hydroxysteroid sulfotransferase; PAPS, 3′-phosphoadenosine-5′-phosphosulfate; PST, phenol sulfotransferase(s); RT-PCR, reverse transcription-polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gels.

Her *et al.* (1996) recently evaluated the expression of EST and HSST in the human jejunal mucosa. They detected significant individual variation in the jejunal expression of both enzymes but found no correlation with gender, age or underlining pathological condition. Given the known role that estrogen plays in breast and uterine cancer development, and given the current interest in the use of estrogen as a hormone replacement therapy, to prevent osteoporosis and cardiovascular diseases, it is of significant interest to evaluate the pattern, mechanism of regulation and physiological implications of EST expression in the human liver, both under normal conditions and in situations where its induction or inhibition might occur. This paper describes the outcome of the first comparative study on the hepatic expression of EST in a number of individual organ donors.

## Materials and Methods

**Human liver samples.** Human livers (five male and five female) were procured by the International Institute for the Advancement of Medicine (Exton, PA). They were donated for transplantation purposes but, for unspecified reasons, were not utilized. All donors were free of known liver disease. Hepatocytes were isolated from fresh liver tissues by perfusion, using a two-step collagenase digestion procedure (Li *et al.*, 1992). After enzymatic dissociation, the hepatocytes were further separated from nonparenchymal cells *via* centrifugation through 30% Percoll. The purified hepatocytes were cryopreserved in 10% dimethyl sulfoxide under liquid nitrogen until analysis.

**Cloning and expression of human EST and PST cDNAs.** To amplify human EST and PST cDNAs by RT-PCR, we purchased a sample of human liver total RNA (from a male subject 40 years old) from Clontech (Palo Alto, CA). First strand cDNA was synthesized from 20 $\mu$g of total RNA, using 400 ng of oligo(dT) 18 and 400 units of Moloney murine leukemia virus reverse transcriptase (Gibco/BRL, Grand Island, NY). The cDNA was ethanol-precipitated and dissolved in 100 $\mu$l of water. We used 2 $\mu$l of this cDNA in subsequent PCR reactions. The PCR reaction mixture consisted of 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 3 mM $MgCl_2$, 0.2 mM each of dNTP, 20 pmol of each primer and 1.25 units of Taq DNA polymerase (Perkin Elmer, Norwalk, CT) in a final volume of 50 $\mu$l. The two oligonucleotide primers used for amplifying the human EST cDNA were 5′-TCA-ACT-AAA-CAG-TGT-ACC-ACA-3′ (upstream) and 5′-ACC-TTC-TTA-GAT-CTC-AGT-TCG-3′ (downstream), and the two primers for human PST were 5′-GAA-TTC-ATG-GAG-CTG-ATC-CAG-GAC-ACC-3′ (upstream) and 5′-TCA-CAG-CTC-AGA-GCG-GAA-CGT-3′ (downstream). They corresponded to the beginning and end sequences of the full coding region in the human EST cDNA (Aksoy *et al.*, 1994) and the phenol-specific form of human PST (P-PST) cDNA (Wilborn *et al.*, 1993), respectively. The amplified cDNA fragments were analyzed on agarose gels (1.2%) and purified with the Promega Wizard PCR purification Kit (Promega Corp., Madison, WI). The human EST cDNA was directly cloned into the eukaryotic expression vector pCR3 (TA Cloning Kit, Invitrogen, San Diego, CA). Sense and antisense orientations of the cDNA were determined by restriction digestion analysis. For the cloning of the human P-PST cDNA, the purified PCR product was first ligated into the pCRII vector (TA Cloning Kit, Invitrogen, San Diego, CA) and digested out with EcoRI enzyme and then subcloned into the pCR3 expression vector. Orientation of the cDNA insert was similarly determined by restriction digestion.

CHO cells (ATCC, Rockville, MD) were cultured in 100-mm culture dishes in MEM $\alpha$-medium containing 10% fetal bovine serum, 2.5 mM HEPES and 2 mM glutamine. Cells were seeded at 40% confluence on day 1 and transfected with EST or P-PST cDNA of either the sense or the antisense orientation the next day. For transfection, 10 $\mu$g of plasmid cDNA per dish was mixed with 60 $\mu$l of Lipofectamine (Gibco/BRL, Grand Island, NY) in 5 ml of serum-free Opti-MEM medium (Gibco/BRL, Grand Island, NY). The mixture was added to the cells, and after 6 h of incubation, 5 ml of normal medium containing 20% FBS was added. Cells were harvested 36 h later for EST or P-PST protein analysis.

**Preparation of CHO cell and human hepatocyte lysates.** Control or cDNA transfected CHO cells and cryopreserved human hepatocytes were washed with phosphate buffered saline and resuspended in 10 mM Tris-HCl, pH 7.5, containing 0.25 M sucrose, 1 mM DTT, 1 mM PMSF and 10% glycerol. Cells were broken up by five short burst of sonication at 4°C. The resulting lysates were centrifuged at 15,000 $\times$ *g* for 20 min and used for EST enzyme activity assays or Western blot analysis. Protein concentrations were determined by the Bradford method with a colorimetric assay kit from Bio-Rad (Richmond, CA).

**Western blot analysis and EST enzyme activity assays.** A polyclonal EST antiserum was developed by using purified bacterially expressed mouse EST as an antigen (Song *et al.*, 1995). The antiserum was affinity-purified on Affigel-15 beads (Sigma, St Louis, MO) to which pure mouse EST protein had been coupled. Cell lysates were electrophoresed on 10% SDS-PAGE (20 $\mu$g per lane), transferred onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH, BA85, 0.45 $\mu$m) and probed with purified EST antiserum. Immunodetections were performed with the enhanced chemiluminescence (ECL) Western blotting detection system from Amersham (Arlington Heights, IL). Sulfotransferase activity was measured with $^3$H-labeled estradiol ([2,4,6,7-$^3$H(N)]-estradiol, 87.6 Ci/mmol, Du Pont New England Nuclear, Boston, MA, final concentration 1.2 nM) in 200 $\mu$l of 200 mM Tris-acetate buffer, pH 7.9, containing 10 mM Mg-acetate, 1.25% Triton X-100, 100 $\mu$M PAPS and an appropriate amount of cell lysates. The reaction was initiated by the addition of substrate and continued for 30 min at 37°C. The reaction mixture was extracted with 2 volumes of dichloromethane and an aliquot of the aqueous phase was counted and taken as a measure for amount of sulfated products.

**Data analysis.** EST protein levels were determined from Western blot analysis by densitometry scanning of the protein bands. Western blot analysis and enzyme activity assays were carried out in a blind fashion, each sample being received and processed with a coded number only. Samples were decoded at the end, and donors were grouped according to gender, age or history of alcohol use; then corresponding groups were compared by Student's *t* test.

## Results

**Human EST antibody characterization.** We previously have developed a polyclonal antibody for mouse EST (Song *et al.*, 1995). Because human and mouse EST are 77% homologous at the amino acid level, we reasoned that this antiserum should be reactive with the human enzyme as well. To confirm this, we expressed human EST in CHO cells and tested the cross-reactivity of the mouse EST antiserum with the expressed EST protein. Full-coding human EST cDNA was first amplified by RT-PCR from total liver RNA with a pair of oligonucleotide primers synthesized according to the published cDNA sequence (Aksoy *et al.*, 1994). The cDNA was cloned into the eukaryotic expression vector pCR3, either in the sense or in the antisense orientation, and was transiently expressed in CHO cells. As shown in figure 1A, a specific protein band at 35 kD, the expected size of human EST protein, was detected on Western blot analysis of CHO cells transfected with the cDNA in the sense orientation. In contrast, no protein was detected in cells transfected with the cDNA in the antisense orientation. This result established that the mouse EST antibody is indeed able to recognize

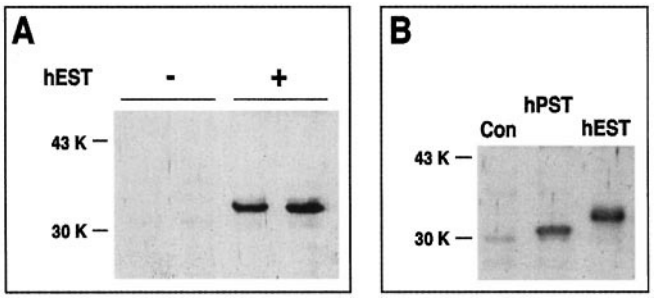


**Fig. 1.** Western blot analysis showing cross-reactivity of the mouse EST antibody with human EST and P-PST expressed in CHO cells. A) Human EST can be recognized by the mouse EST antiserum. −, lysate of CHO cells transfected with human EST cDNA in the antisense orientation; +, lysate of CHO cells transfected with human EST cDNA in the sense orientation. B) The mouse EST antiserum also cross-reacts with human P-PST, but the latter can be distinguished from human EST on SDS-PAGE. Con, untransfected CHO cells; hPST, CHO cells transfected with human P-PST cDNA; hEST, CHO cells transfected with human EST cDNA. Positions of protein molecular weight markers (in daltons) are shown at the left in each panel.

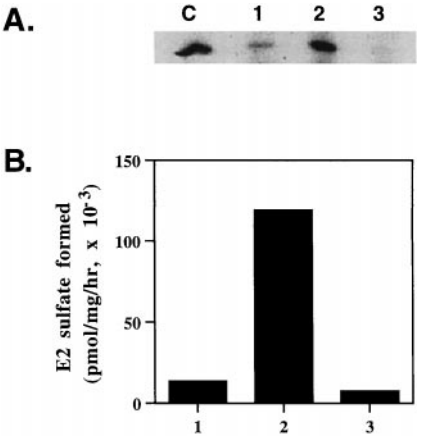


**Fig. 2.** Representative Western blot (panel A) and EST enzyme activity assays (panel B) of three human hepatocyte samples, showing good correlation between protein band intensity and enzyme activity. 1, HH020; 2, HH022; 3, HH023; C, lysate of human EST transfected CHO cells serving as a positive control. Values in panel B represent the average of experiments done in triplicate.

human EST. Because mouse EST and human HSST is only about 25% identical, cross-reactivity of the mouse EST antibody with the human HSST protein is not expected. Although cross-reactivity of our antibody with human PST is a possibility, earlier results from a study by Forbes-Bamforth and Coughtrie (1994) indicated that both the M-PST form and the P-PST form of human PST separated clearly from human EST on SDS-PAGE. To confirm this, we expressed the human P-PST cDNA in CHO cells and carried out Western blot analysis. As shown in figure 1B, we found that although the mouse EST antibody cross-reacted with the expressed human P-PST, human P-PST and EST proteins have clearly different mobilities on SDS-PAGE and can be distinguished easily.

**Western blot analysis of EST expression in the human liver.** Hepatocytes isolated from five female and five male donors were analyzed by Western blot analysis using the above characterized mouse EST antiserum. Their age, their sex and other available background information on the 10 donors are provided in table 1. Figure 2A shows the result of Western blot analysis of three representative human hepatocyte samples. The identity of the EST band on Western blot was confirmed by the use of human EST protein expressed in CHO cells, run at one side of the protein gel as a positive control (fig. 2A). The use of the human EST standard ensured that the observed signals were not due to cross-reactivity of the antiserum with other human sulfotransferases. As discussed above, human M-PST and P-PST have different calculated molecular weights and are expected—and are confirmed, as shown in figure 1B—to separate from EST on SDS-PAGE (Wilborn *et al.*, 1993; Ozawa *et al.*, 1995; Zhu *et al.*, 1993; Otterness, 1992; Forbes-Bamforth and Coughtrie, 1994). Furthermore, measurement of EST activity in the hepatocyte samples from figure 2A showed a good correlation between the enzyme activity and the immunoreactive EST protein band on Western blot (fig. 2B). On the basis of previous enzyme kinetic data (Falany *et al.*, 1994, 1995; Falany and Falany, 1996), the estrogen sulfonating activity of PST or HSST would be expected to be minimal at the concentration of estradiol used in the activity assay (1.2 nm). Thus the Western blot analysis should be both specific and accurate for the detection of human EST in these samples. Levels of EST protein in livers of the 10 donors were quantified by densitometer scanning of the bands on Western blot and are plotted in figure 3. A readily recognizable feature of these data is the marked individual variation in the hepatic expression of EST. For example, subjects HH023 and HH022 differ by more than 25-fold.

**Lack of correlation of the expression of EST with gender and age.** From the data in table 1 and figure 3, it is apparent that the expression of EST in the human liver is not sex-limited as it is in the rat and mouse (Demyan *et al.*, 1992; Song *et al.*, 1997). Group comparison between male and female donors also showed no significant difference in the level of EST expression between the two genders (fig. 4A). In the

TABLE 1
Background information on donors

| Donor ID | Height | Weight (kg) | Sex | Age (years) | Race[a] | Smoking | Alcohol |
|---|---|---|---|---|---|---|---|
| HH013 | 6′ 1″ | 104 | M | 48 | C | no | no |
| HH016 | 5′ 10″ | 79 | M | 46 | C | no | yes |
| HH017 | 6′ 1″ | 70 | F | 59 | C | no | no |
| HH018 | 5′ 4″ | 69 | F | 67 | C | no | yes |
| HH019 | 6′ 2″ | 116 | M | 53 | C | yes | yes |
| HH020 | 5′ 4″ | 104 | F | 65 | C | no | no |
| HH021 | 5′ 11″ | 86 | M | 61 | C | yes | yes |
| HH022 | 5′ 10″ | 63 | F | 76 | C | yes | yes |
| HH023 | 5′ 6″ | 71 | F | 79 | C | no | no |
| HH027 | 6′ 2″ | 84 | M | 44 | C | no | no |

[a] C, Caucasian.

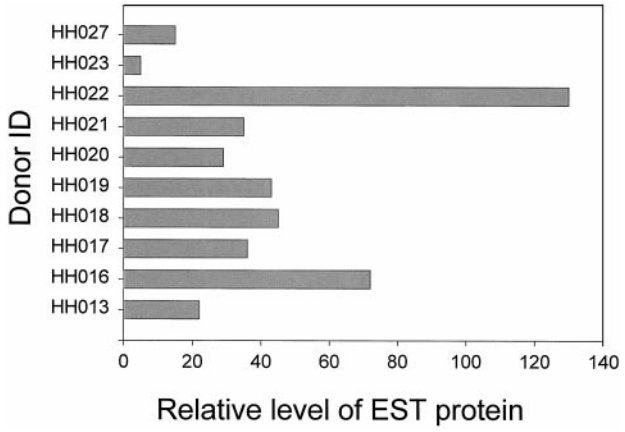


**Fig. 3.** Relative levels of estrogen sulfotransferase in the liver of the 10 donors (determined from 20-$\mu$g of total hepatocyte protein and expressed in arbitrary units). Protein levels were determined by densitometer scanning of band intensity on Western blot. Refer to table 1 for donor information.

rat, EST is expressed in the liver of young and sexually mature males only (Demyan *et al.*, 1992). Whether there is a difference in the hepatic expression of EST between preadolescent and postadolescent human subjects remains to be determined. However, within the age span of the donors (44–79 years old), no correlation between EST expression level and age was observed (fig. 4B). Interestingly, when the donors were grouped according to history of alcohol use, a statistically significant difference emerged between alcohol users and nonusers, with higher EST expression and activity noticed in the livers of the former donor group (fig. 5).

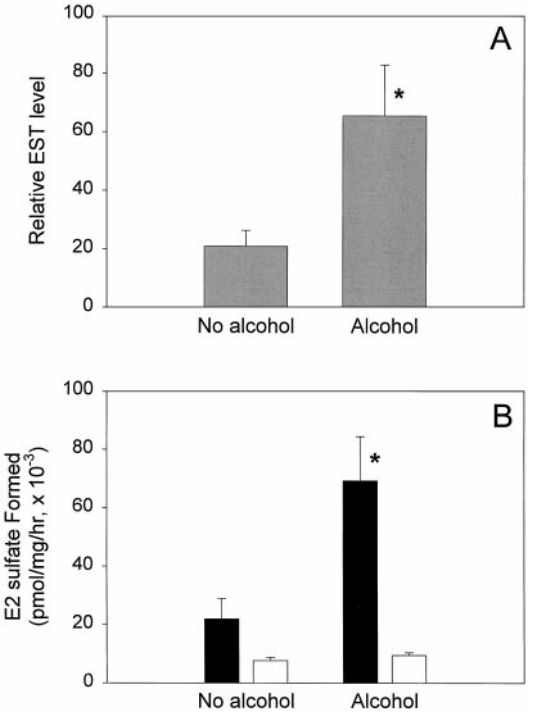


**Fig. 5.** Level of hepatic estrogen sulfotransferase protein (panel A) and activity (panel B) is elevated in alcohol users compared with nonusers (* $P < .05$ by Student's $t$ test). Filled bar graphs in panel B represent average EST activity of the original samples, the open bar graphs that of heat-inactivated samples. Standard error scales indicate interindividual variations ($n = 5$). Activities of each sample were determined in triplicate.

## Discussion

The biological activities of estrogen and other steroid hormones in target tissues depend on two factors: the expression of their cognate receptors and the availability of free, receptor-active ligand in the local environment. *In vivo*, many mechanisms have evolved to regulate the level of active steroid hormones either locally or systemically. These include sex hormone-binding globulins (Petra, 1991) and enzymes involved in the biosynthesis and metabolism of steroid hormones. The importance of these ligand-regulating mechanisms is underscored by the fact that significant effort has been devoted to the development of specific inhibitors for cytochrome P450 aromatase, the ultimate enzyme in the pathway of estrogen biosynthesis and a target for therapeutic intervention in diseases such as breast cancer and benign prostate hyperplasia (Brueggemeier, 1994).

With regard to the metabolism of estrogens, a great deal has been learned about the roles of cytochrome P450 enzymes in the oxidative modifications of estrogens (Martucci and Fishman, 1993). A second significant route for estrogen metabolism is through conjugation reactions such as sulfonation. Hepatic estrogen and other steroid sulfotransferase activities are well known and are usually regarded as part of the phase II drug-metabolizing enzyme systems present in the liver. Recent molecular cloning studies have revealed two important features of the hepatic EST activity (Strott, 1997). One is the existence of an estrogen-specific enzyme that is evolutionarily distinct from the PST and HSST (Weinshiboum, 1997). The other is the high substrate specificity of EST, which has a $K_m$ value comparable to normal levels of plasma estrogen (Falany *et al.*, 1995; Falany and Falany, 1996). The enzyme does not appear to distinguish among the various forms of estrogen, showing avid activity for estradiol and estrone as well as the synthetic ethinylestradiol (Falany *et al.*, 1995; Forbes-Bamforth and Coughtrie, 1994). The dosage for the active estrogen ingredient (often ethinylestradiol)

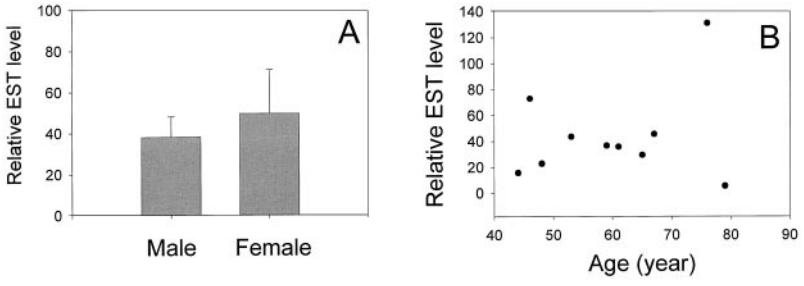


**Fig. 4.** Lack of correlation between hepatic estrogen sulfotransferase protein level and gender or age.

in the commonly used contraceptive pills and hormone replacement therapy is usually at microgram levels (Williams and Stancel, 1996), so the very low $K_m$ value of EST suggests that it may play a major role in the metabolism of both endogenous and exogenous estrogens. However, because of the lack of detailed substrate specificity and enzyme kinetics data, whether PST may, under some circumstances, also contribute to the metabolism of exogenous estrogens remains to be determined. At any rate, there is little doubt that sulfation constitutes a predominant route for *in vivo* estrogen metabolism. Indeed, in a recent study on the metabolism of ethinylestradiol in cultured primary human hepatocytes, sulfation and glucuronidation were the only metabolic transformations observed (Li *et al.*, 1997).

Our demonstration of the lack of gender specificity in the hepatic expression of human EST is in clear contrast with the findings in the rat and mouse, where expression of the enzyme in the liver has been shown to be male-specific (Demyan *et al.*, 1992; Song *et al.*, 1997). In the rat liver, expression of EST is also age-dependent and correlates temporarily with androgen sensitivity. In addition, we previously have found that EST is expressed abundantly in both the rodent and the human testis (Song *et al.*, 1995, 1997). These results suggested that EST might play a more critical role in regulating estrogen activity and homeostasis in males than in females. However, data from the current study imply that there is species variation in the hepatic expression of EST. The lack of gender specificity suggests that EST is likely to play a role in the regulation of estrogen activity in both sexes of human subjects.

The marked interindividual variability in the hepatic EST level indicates that the EST gene is under the sensitive control of genetic and/or environmental factors. In the mouse, expression of EST in the liver is dramatically elevated in the obese and diabetic *ob*/*ob* and *db*/*db* mice (Leiter *et al.*, 1994; Song *et al.*, 1995). However, the *ob*/*ob* or *db*/*db* mutation alone is not sufficient to cause aberrant EST expression. Induction of EST by the two mutations is strain-sensitive and involves interaction with other background genes (Leiter and Chapman, 1989). Whether the *ob* gene product leptin and its receptor play any role in human EST expression is not known. The varied EST expression could also be due to polymorphism in the EST gene allele itself. Our data provide a compelling rationale for future pharmacogenetic studies of this enzyme. Whatever the regulatory mechanism is, the physiological consequence(s) of altered EST expression in the liver may be significant. Increased expression of EST in the liver may change the ratio of free to conjugated estrogens in the plasma and, ultimately, the tissue sensitivity to estrogen. Although sulfonation of a compound increases its polarity and water solubility and facilitates its excretion, the fact that steroid sulfonation is a reversible reaction and blood contains appreciable amounts of circulating estrogen sulfates (Hobkirk, 1985) suggests that the net effect of altered hepatic EST expression on estrogen homeostasis is probably more complex and remains an issue to be clarified. On the other hand, because the liver is well known to be an estrogen target organ, altered hepatic EST expression may have a direct and more predictable effect on the local biological activity of estrogen in this tissue. It is conceivable, for example, that increased EST expression in the liver has a negative impact on the cardioprotective effect of endogenous or supplemented estrogens in women, because much of estrogens' beneficial influence on plasma lipid profile is exercised through their action on the liver (Walsh *et al.*, 1991; Tikkanen *et al.*, 1982; Windler *et al.*, 1980; Landschulz *et al.*, 1996).

A final comment is related to the potential correlation between hepatic EST expression and activity and alcohol consumption. It should be emphasized that although a statistically significant difference was observed between alcohol users and nonusers, further studies involving larger sample sizes would be necessary to establish positively such a connection. Nevertheless, the observation is a rather intriguing one. Exactly how alcohol might regulate the expression of hepatic EST expression and what might be the physiological and pathophysiological consequences of altered EST expression and activity are some of the interesting questions that this study has highlighted. The potential connection between alcohol and sex steroid metabolism is not a new concept. In this regard, it is pertinent to note the well-known feminization syndrome in alcoholic men (Van Steenbergen, 1993). It may also be relevant to refer to the finding that women who are on hormone replacement therapy and who are also alcohol users have a greater risk of developing breast cancer than women on such therapy who are not alcohol users (Golditz *et al.*, 1990). Of special interest is the recently documented study that directly demonstrated that alcohol intake increased free estrogen levels in the blood by 300% in women who were given exogenous estrogen (Ginsburg *et al.*, 1996). If our observation is confirmed by additional studies, the effect of alcohol consumption on hepatic EST activity and expression will constitute a good example of the modulation of EST by environmental factors with considerable physiological implications.

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