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Characterization of Recombinant Human Liver Dehydroepiandrosterone Sulfotransferase with Minoxidil As the Substrate

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ABSTRACT. Biotransformation of xenobiotics and hormones through sulfate conjugation is an important metabolic pathway in humans. The activation of minoxidil, an antihypertensive agent and hair growth stimulator, by sulfation (sulfonation) is carried out by more than one sulfotransferase. Initially only the thermostable form of phenol sulfotransferase was thought to catalyze minoxidil sulfation. We document in this report the new finding that human liver dehydroepiandrosterone sulfotransferase (DHEA ST), an hydroxysteroid sulfotransferase distinct from phenol sulfotransferases, also catalyzes the reaction. To characterize more precisely the activity of DHEA ST toward minoxidil, we used COS-1 cells to express DHEA ST from a human liver cDNA clone. The apparent K_m values for minoxidil and [^{35}S]3′-phosphoadenosine-5′-phosphosulfate were 3.9 mM and 0.13 μM, respectively. The 50% inactivation temperature of the COS-expressed enzyme was 42°, and the IC_{50} value for 2,6-dichloro-4-nitrophenol was 1.4×10^{-4} M. Both the thermal stability behavior and response to DCNP were similar when the cDNA encoded DHEA ST was assayed with DHEA or minoxidil as a substrate. NaCl led to a greater activation of the cDNA-expressed DHEA ST when assayed with DHEA (2.5-fold) than when the same preparation was assayed with minoxidil (1.4-fold). These data indicate that DHEA ST catalyzes the sulfate conjugation of minoxidil. DHEA ST activity present in the human gut and liver would be expected to add to the overall sulfate conjugation of orally administered minoxidil. Thus, DHEA ST activity must be considered when determining the human tissue sulfotransferase contribution to minoxidil sulfation. BIOCHEM PHARMACOL 53;2:215–221, 1997.

KEY WORDS. dehydroepiandrosterone; sulfotransferase; minoxidil; sulfonation; antihypertensive; cytosolic hydroxysteroid sulfotransferase

Sulfate conjugation is an important pathway in the biotransformation of many xenobiotic compounds, catecholamines, as well as steroid and thyroid hormones in humans [1–7]. Minoxidil [6-(1-piperidinyl)-2,4-pyrimidinediamine-3-oxide], a vasodilating agent and hair growth stimulator, is unusual because it is activated by sulfate conjugation [5–8]. Minoxidil sulfate, the active metabolite, affects the arteriolar portions of the circulatory system and is required for the stimulation of hair growth [8–12]. The sulfation of minoxidil is catalyzed by PSTs¶ (EC 2.8.2.1). Both TL and TS forms of PST catalyze the reaction [7, 13–15]. Sulfotransferase activity toward minoxidil has been immunolocalized in the rat hair follicle [16]. During our initial investigation of minoxidil sulfation by human skin PST, we found that TL PST contributed to the reaction [14–15]. We also noted in platelet and skin preparations an apparent enzyme activity toward minoxidil with intermediate thermal stability and greater resistance to inhibitors, behavior similar to DHEA ST [17]. Therefore, we tested expressed human liver DHEA ST in preliminary experiments and found activity toward minoxidil. This was a surprising finding because DHEA ST, an hydroxysteroid sulfotransferase, represents a separate family of sulfotransferases distinct from TL and TS PST [18].

DHEA ST, a cytosolic hydroxysteroid sulfotransferase, has been shown to catalyze the sulfate conjugation of DHEA and steroids such as testosterone and estrone [19, 20]. The enzyme does not sulfate dopamine or *p*-nitrophenol, model substrates for TL PST and TS PST

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¶*Abbreviations:* PST, phenol sulfotransferase; [^{35}S]PAPS, [^{35}S]3′-phosphoadenosine-5′-phosphosulfate; DTT, dithiothreitol; TL, thermolabile; TS, thermostable; DHEA, dehydroepiandrosterone; DHEA ST, dehydroepiandrosterone sulfotransferase; DCNP, 2,6-dichloro-4-nitrophenol; and HSS, high speed supernatant.

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activities, respectively [20, 21]. DHEA ST has been purified from human liver and recently cloned, expressed, and characterized [21, 22]. To confirm our preliminary finding that DHEA ST sulfated minoxidil, we have expressed the human liver DHEA ST cDNA in COS-1 cells and characterized it with minoxidil as the substrate. The use of cDNA expressed enzyme allowed the study of DHEA ST separate from other sulfotransferases often present in tissue preparations. The results confirmed the sulfation of minoxidil by human liver DHEA ST. Because individual variations in hepatic and gut levels of DHEA ST occur [23, 24], the different activities may contribute to variations in responses to orally administered minoxidil. Our finding with DHEA ST emphasized that other sulfotransferases should be considered when evaluating the effects of sulfation on minoxidil metabolism.

MATERIALS AND METHODS

Materials

COS-1 cells were obtained from the American Type Culture Collection (ATCC), Rockville, MD. [^{35}S]PAPS (1.75 to 2.5 Ci/mmol) was purchased from New England Nuclear (NEN) Dupont, Boston, MA. HEPES, BSA, DHEA, DHEA sulfate, and ammonium hydroxide were obtained from the Sigma Chemical Co., St. Louis, MO. DTT was purchased from Calbiochem, La Jolla, CA. Ethyl acetate was obtained from Mallinckrodt, Paris, KY. The human liver DHEA ST cDNA was a gift from D. M. Otterness and R. M. Weinshilboum. Minoxidil and minoxidil sulfate were gifts from the Upjohn Co., Kalamazoo, MI.

Expression in COS-1 Cells

COS-1 cells (an SV40 transformed simian cell line) were maintained in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum [25]. Cells were seeded in 60 mm^2 dishes at 1×10^6 cells/dish, and the following morning the cells were transfected with the human liver DHEA ST cDNA cloned into the *Eco*RI site of plasmid p91023(B), by the calcium phosphate method [21, 26, 27]. The transfected cells were maintained at 37° for 72 hr prior to harvesting.

Processing Transfected COS-1 Cells

The growth medium was removed, and the cells were washed with PBS. The cells were removed with a rubber policeman, resuspended in PBS, and pelleted by centrifugation at 150 *g* for 10 min. The supernatant was removed, and the cells were resuspended in 2.0 mL of homogenate buffer (5 mM potassium phosphate, pH 7.5, 2.5 mM DTT, 1.25 mM EDTA). The cell suspension was homogenized for 15 sec with a Polytron homogenizer and centrifuged at 100,000 *g* for 1 hr at 4°. The resulting HSS was mixed 1:1 (v/v) with a 5 mM potassium phosphate buffer, pH 7.5, that contained 2.5 mg/mL BSA, and aliquots of 700 μL were stored at −75° until assay.

Sulfotransferase and Protein Assays

DHEA ST activity was assayed by the methods of Foldes and Meek [28] as modified by Hernandez *et al.* [17]. Initially, the assay for the cDNA expressed DHEA ST was optimized with regard to protein concentration, reaction pH, and substrate concentrations. The HSS was diluted 80-fold with 5 mM potassium phosphate buffer, pH 7.5, containing 0.625 mg/mL BSA. The final assay concentration of DHEA was 2.5 μM in active samples, whereas blank samples contained no substrate. The cDNA expressed DHEA ST activity assayed with minoxidil was measured by the method of Johnson and Baker [29] as modified by Kudlacek *et al.* [14]. Prior to assay, the HSS was diluted 80-fold with 20 mM HEPES, pH 7.5, containing 5.0 mM DTT and 0.625 mg/mL BSA. Minoxidil (14.6 mM final concentration) was used in active samples, whereas blank samples contained the 0.82 N HCl vehicle, which was used to dissolve the minoxidil. Two active samples were assayed at each test point, and [^{35}S]PAPS at a final concentration of 0.4 μM was used in both assays. Control cells transfected with the vector alone and mock transfected controls contained minimal DHEA ST activity (<1.8%), a finding similar to nontransfected COS-1 cell preparations (Table 1). The mean activity detected in nontransfected control samples was subtracted from the corresponding mean activity in transfected samples to provide a net activity per milligram of protein devoid of endogenous sulfotransferase activity. Total protein in each sample was determined by the method of Bradford [30] with BSA as the standard. The net enzyme activity was expressed as units of activity per milligram of protein. One unit of enzyme activity represented 1 nmol of sulfated product formed in 1 hr of incubation at 37°.

Thermal Stability

Thermal stability was tested by the methods of Reiter *et al.* [31] as modified by Anderson and Liebentritt [32]. Briefly, the thawed HSS preparations were diluted with an equal

TABLE 1. Sulfotransferase activities in COS-1 cells

	Activity (U/mg protein)	
	Substrate	
Procedure	DHEA*	MNX*
Transfected cDNA†	2.86	2.54
Nontransfected	0.125 (4.4%)	0.126 (5.0%)
Vector alone transfected	0.003 (0.1%)	0.0002 (<0.1%)
Mock transfected (no DNA)	0.048 (1.7%)	0.017 (0.7%)

* Numbers in parentheses indicate percentage of transfected activity.

† Data from Fig. 1A and the experiment using various DHEA concentrations described in Results. See text for details.

volume of the potassium phosphate dilution buffer and then were heated for 15 min at either 35°, 37°, 39°, 41°, 43°, 45°, or 47° prior to assay with minoxidil or DHEA. Unheated aliquots were kept at 4° as controls.

Reaction Product Identification by HPLC

Minoxidil sulfate was identified by HPLC with a C18 μBondapak reverse phase column by the methods of Johnson *et al.* [5, 6] as modified by Kudlacek *et al.* [14]. Blank and active assay samples were eluted at 1.0 mL/min with the mobile phase buffer that consisted of 1.0 mL/L trifluoroacetic acid and 30% acetonitrile, and were collected in 0.5-mL fractions. DHEA sulfate was identified by HPLC with the same conditions and elution parameters as the minoxidil sulfate except that the mobile phase buffer was 0.1 M ammonium acetate, pH 5.0, with 50% acetonitrile.

Data Analysis

Apparent K_m values were calculated by the direct linear plot method [33] with the Enzpack 3 program (Elsevier-Biosoft). Temperatures at which 50% of the net activities remained and IC_{50} values for DCNP and NaCl were determined using a curve-fitting program (in Prism, Graphpad Software, San Diego, CA).

RESULTS

Effects of Varying the Substrate Concentrations

The expressed human liver cDNA DHEA ST HSS was used to determine the apparent K_m values. Final concentrations of minoxidil that were tested ranged from 0.014 to 18.25 mM. Activity was detected only with minoxidil concentrations from 0.91 to 18.25 mM. The apparent K_m value for minoxidil was 3.9 mM (Fig. 1A), and the value for DHEA was 0.7 μM (data not shown). The value for minoxidil was similar to the previously reported apparent K_m values obtained with partially purified human platelet PST activities and expressed human liver TL PST activity assayed with minoxidil as shown in Table 2 [14, 15]. The apparent K_m value for DHEA was similar to a value of 2.6 μM obtained by Aksoy *et al.* [34] with cDNA expressed human liver DHEA ST assayed with DHEA. The apparent K_m values for [^{35}S]PAPS determined with minoxidil and DHEA as the constant substrates were 0.13 and 0.01 μM, respectively (panels B and C of Fig. 1). The former value was similar to apparent K_m values reported for [^{35}S]PAPS with human platelet and skin PST activities assayed with minoxidil (0.24 and 0.15 μM, respectively) [14]. The latter apparent K_m for [^{35}S]PAPS with DHEA as the constant substrate (Fig. 1C) was the same as the value reported by Wood *et al.* [35] with human liver cDNA expressed DHEA ST.

When the expressed DHEA ST preparation was assayed with 60 μM dopamine, a model substrate for TL PST, no activity was observed. This finding indicated that no detectable endogenous TL PST activity was present. A small amount of endogenous TS PST activity that represented 1.8% of the DHEA ST activity was observed when the expressed material was assayed with 4.0 μM *p*-nitrophenol. The potential effect of this activity was removed by sub-

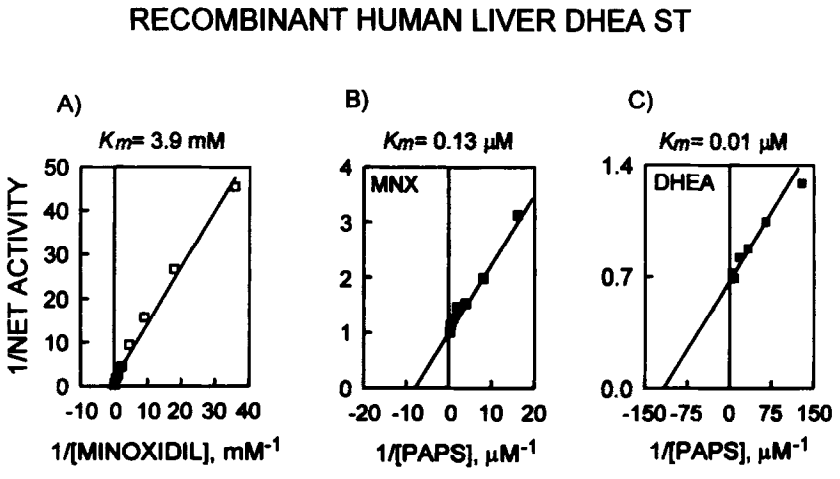

FIG. 1. Double-reciprocal plots of recombinant DHEA ST activity obtained with varying concentrations of co-substrates. (A) Plot with minoxidil as the variable substrate and [^{35}S]PAPS as the constant substrate. Activity at V_{max} was 2.54 U/mg protein and the corresponding non-transfected control was 5.0% of the activity. (B) Plot with [^{35}S]PAPS (PAPS) as the variable substrate and minoxidil (MNX) as the constant substrate. Activity at V_{max} was 1.03 U/mg protein, and the corresponding nontransfected control was 4.1%. (C) Plot with PAPS as the variable substrate and DHEA as the constant substrate. Activity at V_{max} was 0.94 U/mg protein, and the corresponding nontransfected control was 0%. Apparent K_m values are given at the top of each panel. Net activity was expressed as U/mg protein. Each point was assayed in duplicate.

TABLE 2. Apparent K_m values for minoxidil with expressed human liver and partially purified human platelet sulfotransferases

	K_m (mM)
Expressed liver cDNA	
DHEA ST	3.9
TL PST	2.8*
Partially purified platelet	
TS PST	1.49†
TL PST	2.96†

* Ref. 15.
† Ref. 14.

traction of the control COS-1 cell activity measured with minoxidil.

Effects of Temperature

Thermal stability is an important indicator of a difference in protein structure, and is useful in differentiating many human sulfotransferases [14, 31]. It was expected that if the same enzyme used DHEA and minoxidil as substrates, then similar thermal stability behaviors would be seen in assays with these two substrates. Thermal stability patterns of the cDNA expressed DHEA ST with minoxidil and DHEA as substrates were nearly identical, yielding 50% inactivation temperatures at 42° (Fig. 2). Thus, these results supported the conclusions that human DHEA ST used minoxidil as a substrate, and indicated that the minoxidil assay did not alter thermal stability.

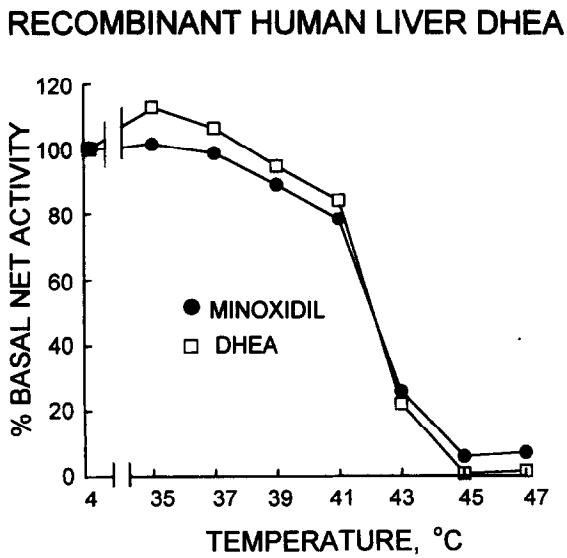

FIG. 2. Thermal stability of recombinant human liver DHEA ST with minoxidil and DHEA as substrates. The DHEA ST activity for transfected COS-1 cells is expressed as a percentage of the basal (unheated) net activity. The 50% inactivation temperature was 42° with both substrates. The cpm/mg protein values at basal activity were 1850 with minoxidil and 1970 with DHEA. The basal activities of the nontransfected controls with minoxidil and DHEA were 6.4 and 0% of the transfected samples, respectively. Each point was assayed in duplicate.

Effects of DCNP

DCNP has been shown to inhibit DHEA ST activity with an IC_{50} value of 4.0×10^{-5} M when assayed with DHEA as the substrate [34]. Therefore, it was of interest to determine whether DCNP also had this effect on DHEA ST assayed with minoxidil. The cDNA expressed DHEA ST activity assayed with DHEA and minoxidil in the presence of DCNP yielded similar IC_{50} values for DCNP of 8.1×10^{-5} and 1.4×10^{-4} M, respectively (Fig. 3). The fact that DCNP inhibition was similar with both substrates further supported the conclusions that human DHEA ST activity sulfated minoxidil and that a single enzyme catalyzed the reactions.

Effects of NaCl

NaCl at concentrations of 50 and 200 mM inhibits TS PST and TL PST activities, respectively [14, 15]. These NaCl concentrations lead to activation of DHEA ST activity [34–36], with a 1.6-fold rise reported by Aksoy *et al.* [34]. NaCl activation of enzyme activities has been seen with other steroid sulfotransferases, and NaCl has been incorporated into the assays of these enzymes to enhance their activities [36]. Therefore, to confirm that the activity tested was neither TS PST nor TL PST, and to determine whether a similar activation took place when DHEA ST sulfated minoxidil, the expressed DHEA ST material was assayed in the presence of various concentrations of NaCl. The cDNA expressed DHEA ST activity measured with both DHEA and minoxidil was activated by the addition of NaCl (Fig. 4). The activity with DHEA rose 2.5-fold with the addition of 200 mM NaCl, whereas activity with minoxidil rose approximately 1.4-fold at 25 mM NaCl, and then was 50% inhibited at 173 mM NaCl (Fig. 4). There was no apparent inhibition of DHEA ST below basal activity when assayed with DHEA (Fig. 4).

Identification of Reaction Products by HPLC

Reverse-phase HPLC was used to identify the reaction product of DHEA ST HSS measured with DHEA and minoxidil. The net radioactivities that co-eluted with known DHEA sulfate and minoxidil sulfate were 99 and 94%, respectively. The patterns of elution and the longer retention of minoxidil sulfate compared with minoxidil were similar to results reported by Johnson *et al.* [5]. The results indicated that DHEA ST was expressed in the COS-1 cells, and they supported our data that demonstrated that DHEA ST was able to catalyze the sulfation of minoxidil.

DISCUSSION

Initially, minoxidil was thought to be sulfated only by TS PST [13], but our recent studies have shown that TL PST

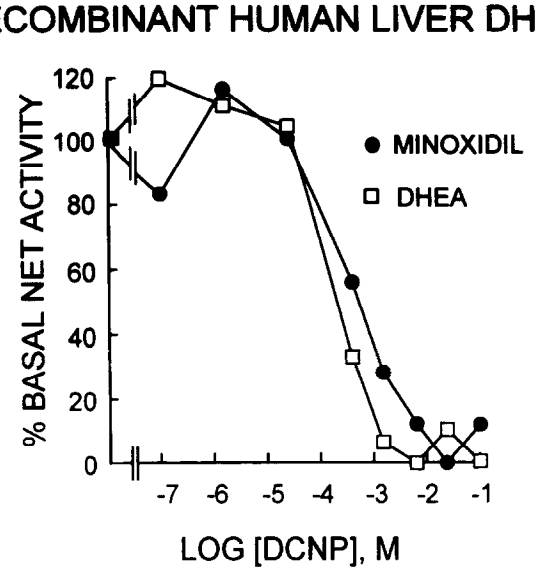

FIG. 3. Effects of DCNP on recombinant DHEA ST activity assayed with minoxidil and DHEA. The graph shows the enzyme activity in COS-1 transfected cells expressed as a percentage of the basal net activity (sample containing no inhibitor). The IC_{50} values for DCNP were 8.1×10^{-5} and 1.4×10^{-4} M with DHEA and minoxidil, respectively. The cpm/mg protein values at basal activity for minoxidil and DHEA were 930 and 1510, respectively. The basal nontransfected controls were 5.8 and 0% of the transfected activities with minoxidil and DHEA, respectively. Each point was assayed in duplicate.

also sulfates minoxidil [14, 15]. We now report the unique observation that DHEA ST is also capable of sulfating minoxidil. This is an important finding because it documents the ability of an hydroxysteroid sulfotransferase to contribute to the sulfation of minoxidil, a reaction previously attributed only to PSTs. Because the levels of hepatic DHEA ST are substantial and jejunal DHEA ST is present, the enzyme may have an important impact on activation of orally administered minoxidil through sulfation. The availability of cDNAs for this enzyme has made possible detailed studies of DHEA ST with minoxidil as the substrate.

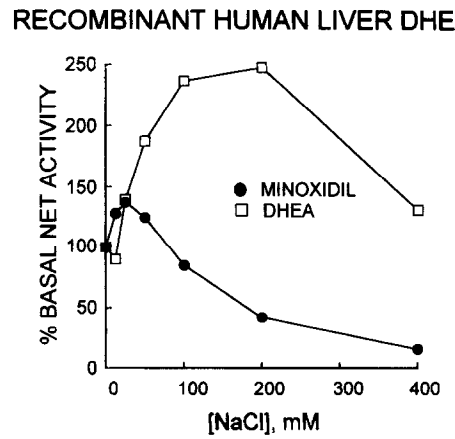

FIG. 4. Effects of NaCl on recombinant DHEA ST activity assayed with minoxidil and DHEA. The graph shows the enzyme activity in transfected COS-1 cells expressed as a percentage of the basal net activity (sample containing no NaCl). Activation with both substrates occurred with NaCl. There was no inhibition below basal level when activity was assayed with DHEA beyond 25 mM NaCl. The IC_{50} of NaCl with minoxidil as the substrate was 173 mM. The cpm/mg protein values at baseline with minoxidil and DHEA were 1400 and 2890, respectively. The basal nontransfected controls with minoxidil and DHEA were 9.0 and 1.7% of the transfected activities, respectively. Each point was assayed in duplicate.

In these studies, we have characterized the sulfation of minoxidil by DHEA ST that was expressed from a human liver DHEA ST cDNA clone in COS-1 cells. This approach has allowed us to investigate a DHEA ST preparation essentially devoid of other sulfotransferases. The behavior of the recombinant DHEA ST assayed with either minoxidil or DHEA as substrate was essentially the same with respect to thermal stability and inhibition by DCNP. Furthermore, the HPLC results indicated that the recombinant DHEA ST produced authentic minoxidil sulfate. Of interest was the finding that the results were quite different when the activity of the expressed DHEA ST was assayed with either DHEA or minoxidil as the substrate in the presence of increasing concentrations of NaCl. The NaCl activation of DHEA ST with DHEA as the substrate and the attenuated activation at higher NaCl concentrations were typical of behaviors of cDNA expressed DHEA ST [34]. Conversely, when the DHEA ST preparation was assayed with minoxidil in the presence of increasing NaCl concentrations, the activity continued to be activated at 50 mM NaCl, but then decreased with higher amounts of NaCl (Fig. 4). The decrease in activity represented not only attenuation, but also extensive inhibition below baseline activity. This inhibition might have been an effect of the minoxidil assay with its different buffers and assay conditions, or minoxidil sulfate itself might have induced nonenzymatic covalent protein sulfation that altered DHEA ST behavior [37].

The fact that an hydroxysteroid sulfotransferase catalyzes minoxidil sulfation is intriguing. Studies of the common molecular domains of these sulfotransferase cDNAs may help to explain the ability of diverse enzymes to utilize minoxidil as a substrate. There are large individual variations in the levels of human liver DHEA ST activity, and the amount of this hepatic enzyme activity is substantial [23]. Variable levels of the quantity of immunoreactive DHEA ST protein also have been found in human jejunum

[24]. The levels of activity and the degree of variability may be factors that contribute to variations in individual responses to orally administered and circulating minoxidil.

An apparent K_m value of 3.9 mM for minoxidil with expressed DHEA ST was similar to values of 1.49 and 2.96 mM obtained with partially purified platelet TS PST and TL PST [14], respectively, and 2.8 mM with expressed human liver TL PST [15] (Table 2). Similar values were found with HSS preparations of human skin TL and TS PST [14]. Whether these higher apparent K_m values accurately reflect the potential physiologic function of the enzyme is not known. Broken-cell preparations alter the intracellular microenvironment of the enzyme, and this may affect accurate measurement of activity. Expressed sulfotransferases assayed *in vitro* suffer from a similar distortion of the physiologic conditions for optimal enzyme activity. Our studies of partially purified and expressed sulfotransferases with minoxidil concentrations as low as 12 μM did not reveal an enzyme activity with a higher affinity for minoxidil. The findings suggest that DHEA ST activity, as well as TL PST and TS PST activities, should be taken into account when attempting to determine the regulation of minoxidil sulfation. The presence of both TL and TS PST activities in human scalp skin is known, as are the correlations of these activities with their respective platelet levels [14]. The importance of inter-individual variations in human skin phenol sulfotransferases and their potential effects on individual hair growth responses to topical minoxidil remain to be shown. Preliminary studies of human scalp skin HSS pools with an assay that has not been optimized have shown low levels of DHEA ST activity (Kudlacek PE and Anderson RJ, unpublished data). It is premature to predict a contribution of putative skin DHEA ST activity to the metabolism of topical minoxidil.

In summary, we have confirmed that human liver DHEA ST activity sulfate conjugates minoxidil. The finding concurs with the observations that multiple sulfotransferases with different kinetic and biochemical properties will catalyze the sulfation of a single substrate, whether the substrate is a xenobiotic, catecholamine, or steroid or thyroid hormone. The results point out the complex, overlapping enzymatic mechanisms that contribute to the sulfate conjugation of substrates. The situation is similar to the sulfate conjugation of triiodothyronine, a hormone that is sulfated by at least three sulfotransferases with different kinetic properties: TS PST [38, 39]; TL PST [38]; and DHEA ST [39, 40]. To begin to understand the individual contribution and regulation of each sulfotransferase, careful biochemical characterization of each enzyme with the substrates of interest is essential.

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