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SULFATION OF MINOXIDIL BY HUMAN LIVER PHENOL SULFOTRANSFERASE

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Abstract—The *N,O*-sulfate of minoxidil (Mnx) is the active agent in producing the vasodilation and the hair-growth stimulating responses observed with Mnx treatment. In this report, Mnx sulfation activity was assayed in cytosol prepared from several normal human livers, and Mnx sulfation was shown to correlate significantly with the activity of the phenol-sulfating form of phenol sulfotransferase (P-PST) activity in the same livers. No correlation was observed between Mnx sulfation and the dopamine or dehydroepiandrosterone (DHEA) sulfotransferase activities present in human liver. Mnx sulfation also copurified with P-PST activity during the purification of P-PST from human liver. During the purification procedure, Mnx and *p*-nitrophenol sulfotransferase (P-PST) activities were resolved from the dopamine and DHEA sulfation activities catalyzed by the monoamine-sulfating form of phenol sulfotransferase (M-PST) and DHEA sulfotransferase respectively. Also, purified DHEA sulfotransferase was not capable of sulfating Mnx, and no data were obtained to indicate that Mnx is a substrate for M-PST. *p*-Nitrophenol, a substrate for P-PST, was demonstrated to be a competitive inhibitor of Mnx sulfation catalyzed by purified P-PST when Mnx was the variable substrate. These results indicate that Mnx is sulfated and, therefore, bioactivated by P-PST in human liver.

Minoxidil [6-(1-piperidinyl)-2,4-pyrimidinediamine-3-oxide, Loniten,® Rogaine,® is a potent vasodilator and is used therapeutically as a hypotensive agent [1, 2]. A common side-effect observed in patients following the use of orally administered minoxidil (Mnx) is an increased incidence of hypertrichosis, increased hair growth [3]. This observation has led to the use of topically administered Mnx for the treatment of alopecia androgenica, male pattern baldness [4]. Several studies [2, 5–7] have indicated that a metabolite of Mnx, Mnx-sulfate, is the active compound eliciting the vasodilator effect and hair-growth induction effect. The actual active form or metabolite of Mnx which is responsible for the different therapeutic effects of Mnx is the *N,O*-sulfate ester [2].

Johnson and Baker [8] have reported that Mnx is sulfated by phenol sulfotransferase (PST) activity present in human platelets; however, it was not determined which of the two forms of PST present in platelets is responsible for the conjugation of Mnx. Platelets contain two distinct forms of PST: M-PST and P-PST [9–11]. M-PST is responsible for the sulfation of monoamines, such as dopamine, whereas P-PST is capable of conjugating small phenols and structurally related neutral compounds. Johnson and Baker [8] have also suggested that the levels of Mnx sulfation in platelets are insufficient to account for the rate of Mnx sulfation observed in humans. Therefore, other tissues, such as human liver, may be important in the metabolism and bioactivation of Mnx.

Falany *et al.* [12] have reported that cytosol prepared from several normal human livers possesses a specific activity for P-PST 10- to 30-fold greater than that observed in platelets. Also, human liver cytosol contains relatively high levels of immunoreactive P-PST as compared to the levels of M-PST [13]. This report indicates that human liver possesses high levels of Mnx sulfation activity as compared to platelets [8] and demonstrates that Mnx-ST activity copurified with the P-PST form of sulfotransferase in normal human liver.

MATERIALS AND METHODS

Materials. 3′-Phosphoadenosine-5′-phosphosulfate (PAPS) was purchased from Pharmacia (Piscataway, NJ). DEAE-Sepharose Cl-6B, *p*-nitrophenol (PNP), dehydroepiandrosterone (DHEA), and 3′,5′-diphosphoadenosine (PAP)-agarose were obtained from the Sigma Chemical Co. (St. Louis, MO). [^{35}S]PAPS (1.1 Ci/mmol) and [1,2,6,7-^{3}H]DHEA (100 Ci/mmol) were obtained from New England Nuclear (Boston MA). Mnx was a gift of The Upjohn Co. (Kalamazoo, MI). PM30 membranes were obtained from the Amicon Corp., (Danvers, MA). All other chemicals were of reagent grade quality.

Preparation of liver cytosol. Human liver specimens were obtained from the Organ Procurement Program at the University of Rochester at the time of recovery of other tissues for transplantation. The livers were chilled on ice immediately after removal from the donor and were subsequently frozen at −70° within 30 min of recovery. Prior to the chromatographic procedures, liver samples were thawed in 10 mM triethanolamine, pH 7.5, with 1.5 mM dithiothreitol and 10% glycerol (TEA buffer), minced with scissors, and then homogenized with a

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Brinkmann Polytron apparatus. The liver homogenates were centrifuged at 12,000 *g* for 15 min at 4°, and the supernatant fractions were recovered and centrifuged at 100,000 *g* for 1 hr. Liver cytosols were then removed, filtered through several layers of sterile cheesecloth, divided into aliquots, and stored at −70°. The Bio-Rad protein assay, originally developed by Bradford [14], was used to estimate protein concentrations using bovine serum albumin as a standard.

Sulfotransferase assays. Mnx sulfotransferase activity was determined as described by Johnson and Baker [8]. The assay mixture contained 240 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer, pH 7.3, 7 mM minoxidil, and 7 mM dithiothreitol unless otherwise noted. Reactions were started by the addition of [^{35}S]PAPS to a final concentration of 10 μM and were incubated for 10 min at 37°. The reactions were terminated by the addition of 1 mL of ethyl acetate and 0.4 mL of 1 M ammonium hydroxide. The reaction mixtures were then vortexed for 15 sec, and centrifuged at 800 *g* to separate the organic and aqueous phases; the radioactivity in 0.5 mL of the ethyl acetate phase, which contains the sulfated Mnx, was determined by scintillation spectrometry. This extraction procedure takes advantage of the fact that Mnx sulfate is more hydrophobic than Mnx and is extracted into the organic phase [2]. Highly polar sulfate esters, such as DHEA sulfate, PNP sulfate and dopamine sulfate, remain in the aqueous phase.

Phenol sulfotransferase activity was assayed using the barium precipitation method of Foldes and Meek [15]. The reaction mixture contained 50 mM sodium phosphate buffer, pH 6.8, and either 4 μM PNP or 10 μM dopamine. The dopamine assays also contained 1 mM pargyline. The reactions were initiated by the addition of the [^{35}S]PAPS (approx. 0.05 μCi/nmol) to a final concentration of 10 μM in a final reaction volume of 0.25 mL. Reactions were terminated with the addition of 50 μL each of 0.1 M solutions of barium acetate, barium hydroxide, and zinc sulfate. The precipitate was removed by centrifugation and the supernatant fractions were transferred to microcentrifuge tubes. The supernatant fractions were then reprecipitated with barium acetate, barium hydroxide and zinc sulfate, and centrifuged at 12,000 *g* for 3 min. The radioactivity in the supernatant fractions was determined by scintillation spectrometry. DHEA sulfotransferase activity was determined following the method of Falany *et al.* [12] using [^{3}H]DHEA as the sulfate acceptor.

Purification of human liver sulfotransferase activities. The purification procedure for Mnx sulfotransferase activity was performed essentially as described by Falany *et al.* [12] for the purification of DHEA-sulfotransferase. Samples of human liver cytosol were thawed in TEA buffer on ice, and all purification procedures were performed at 4°. Liver cytosol (700 mg) was applied to a DEAE-Sepharose Cl-6B column (2 × 16 cm) previously equilibrated in TEA buffer. The column was then washed with 4 column volumes of TEA buffer containing 100 mM NaCl. Sulfotransferase activities were eluted from the anion-exchange column with a 100–225 mM NaCl gradient in TEA buffer. Fractions containing high levels of Mnx and PNP sulfotransferase activity were pooled and concentrated using an ultrafiltration stirred cell with a PM30 membrane.

The concentrated fractions from the anion-exchange column were then added very slowly to a PAP-agrose affinity column (0.7 × 10 cm) previously equilibrated in TEA buffer. The affinity column was washed with 6–7 column volumes of TEA buffer containing 50 mM NaCl, and the sulfotransferase activities were eluted with a linear gradient of PAPS (0–20 μM) in the same buffer. Sulfotransferase activity is assayed in 5-μL aliquots of the fractions collected during the affinity procedure in order to reduce the amount of cold PAPS added to the assays. The PAPS gradient has been reported previously to resolve DHEA and PNP sulfotransferase activities during the affinity chromatography procedure [12].

RESULTS

Mnx sulfation was observed in cytosol prepared from several different normal human livers. Therefore, to establish appropriate reaction conditions for the determination of Mnx sulfation activity in human liver cytosol, the apparent K_m values for Mnx and PAPS were determined using Mnx and PAPS concentrations between 0.18 and 7.0 mM and 0.05 and 5 mM respectively. The K_m values for Mnx and PAPS using human liver cytosol were estimated to be 0.89 mM and 0.5 μM respectively (data not shown). Next, using the assay conditions described in Materials and Methods, Mnx sulfation in human liver cytosol was demonstrated to be linear with respect to both protein concentration and time (Fig. 1).

Mnx sulfation activity and PNP sulfation catalyzed by P-PST were assayed in cytosol prepared from several normal human livers to determine if the levels of Mnx sulfation in the different livers were associated with the levels of P-PST activity. Figure 2 illustrates that the levels of P-PST and Mnx sulfotransferase activities displayed a high degree of correlation ($r = 0.98$, $P > 0.01$) in cytosol prepared from seven normal human livers. In contrast, a correlation of $r = -0.21$ was observed between dopamine, a specific M-PST substrate, and Mnx sulfotransferase activities in these livers (data not shown).

To determine if P-PST, which catalyzes PNP sulfation, was also capable of sulfating Mnx, the elution of Mnx sulfation activity was followed through the purification of P-PST. Since DHEA sulfotransferase, M-PST and P-PST activities have been shown previously to elute during DEAE-Sepharose Cl-6B chromatography at different salt concentrations [12, 13], this anion exchange resin was used to determine if Mnx sulfation coeluted with either P-PST, M-PST or DHEA sulfotransferase activities. Figure 3 shows that Mnx sulfation activity coeluted from the anion-exchange column with P-PST activity. In addition, Fig. 4 shows that the Mnx sulfation activity eluted from the DEAE-Sepharose column at a lower NaCl concentration than dopamine sulfation activity, indicating that Mnx is apparently not a substrate for human liver M-PST activity. Fractions from the DEAE-Sepharose chromatography procedure which

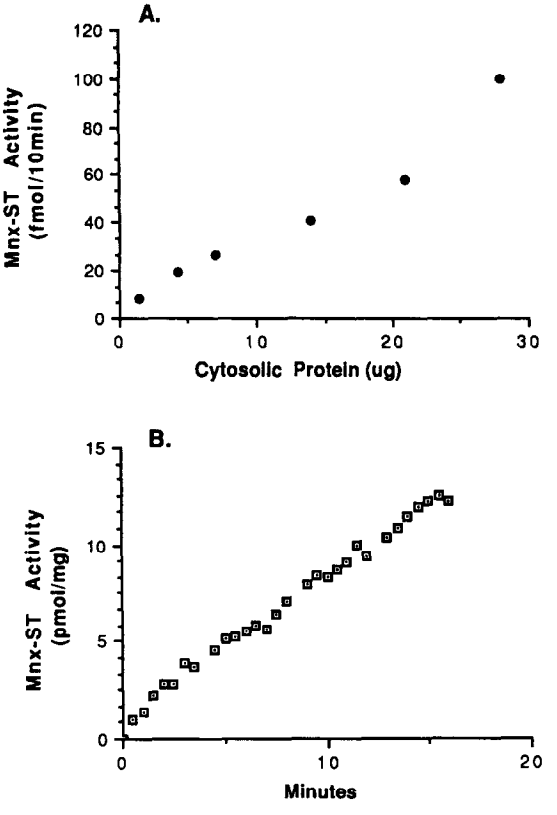

Fig. 1. Linearity of Mnx sulfation with respect to liver cytosol protein and incubation time. Panel A: Cytosol was prepared from human liver 6 (HL6) as described in Materials and Methods. Reactions contained 6.4 mM Mnx and 10 μM PAPS and were incubated at 37° for 10 min. Panel B: Aliquots (170 μL) were removed at specified times from a Mnx sulfotransferase assay containing 6.4 mM Mnx, 20 μM PAPS and 750 μg/mL cytosolic protein (HL6), the reaction was stopped by the addition of 1 mL ethyl acetate, and Mnx sulfate was determined as described in Materials and Methods.

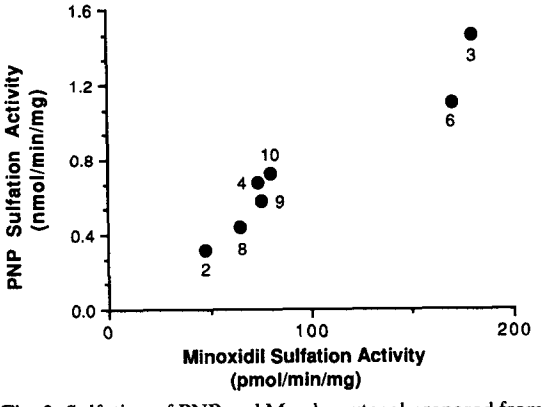

Fig. 2. Sulfation of PNP and Mnx by cytosol prepared from different human livers. Cytosol was prepared from normal human livers, and Mnx and PNP sulfotransferase activities were assayed as described in Materials and Methods. The sex and age in years of the donors were: HL2 (F,6), HL3 (F,56), HL4 (M,55), HL6 (M,56), HL8 (M,41), HL9 (F,41) and HL10 (F,66).

contained high levels of Mnx and PNP sulfotransferase activity were pooled, concentrated by ultrafiltration, and applied to a PAP-agarose affinity column. Figure 5 demonstrates that PNP and Mnx sulfotransferase activities coeluted from the PAP-agarose affinity column during the PAPS gradient.

Table 1 demonstrates that the ratio of PNP sulfotransferase activity to Mnx sulfotransferase activity remained constant during the purification procedure for P-PST. The total recovery of both PNP and Mnx sulfotransferase activities was 21%, and a purification of approximately 500-fold was obtained as compared to these sulfotransferase activities in liver cytosol. The affinity-purified preparation of P-PST activity contained no dopamine sulfotransferase activity and only low contaminating levels of DHEA sulfotransferase. Pure DHEA sulfotransferase was also not observed to sulfate Mnx.

The ability of PNP to inhibit Mnx sulfation catalyzed by affinity-purified P-PST was also tested. Figure 6 shows that increasing concentrations of PNP readily inhibited Mnx sulfation, and this inhibition appeared to be via a competitive mechanism with respect to Mnx (inset, Fig. 6). Using inhibitory concentrations of PNP from 0.25 to 1 μM, a K_i of 0.45 was calculated for PNP inhibition of Mnx sulfation. The K_i for PNP is very similar to the K_m (0.43 mM) reported for PNP sulfation catalyzed by P-PST activity [16]. Dopamine, added up to a final concentration of 20 μM, did not inhibit Mnx sulfation activity in liver cytosol (data not shown).

Since M-PST activity is very low relative to P-PST activity in liver, Mnx sulfation catalyzed by M-PST may be masked by the high levels of P-PST activity. To confirm that M-PST does not sulfate Mnx, the M-PST and P-PST activities in platelet cytosol were resolved by DEAE-Sepharose Cl-6B chromatography as described by Heroux and Roth [17]. Platelets possess 2- to 3-fold higher levels of M-PST activity than P-PST activity [17]. As observed with liver, the Mnx sulfotransferase activity coeluted with the P-PST activity and no Mnx sulfation was observed in fractions containing only M-PST activity (data not shown).

DISCUSSION

Mnx sulfate has been reported to be the active agent in causing the antihypertensive [2, 5] and hair-growth stimulating effects [7] observed in humans treated with Mnx. In this report, human liver has been identified as a source of high levels of Mnx sulfation activity. Whereas, in most instances, sulfate conjugation results in an increase in the hydrophilicity of a compound, Mnx *N,O*-sulfate is more hydrophobic than Mnx [2]. High levels of Mnx sulfation in liver, therefore, may serve as an important mechanism for maintaining tissue and circulating levels of Mnx sulfate. Also, P-PST activity [18] and immunoreactivity [13] have been detected in a number of different human tissues, indicating that Mnx sulfation is widespread throughout the body.

The data in this report indicate that P-PST is responsible for most, if not all, of the sulfation of Mnx in human liver cytosol. No evidence was observed for the sulfation of Mnx by either M-PST

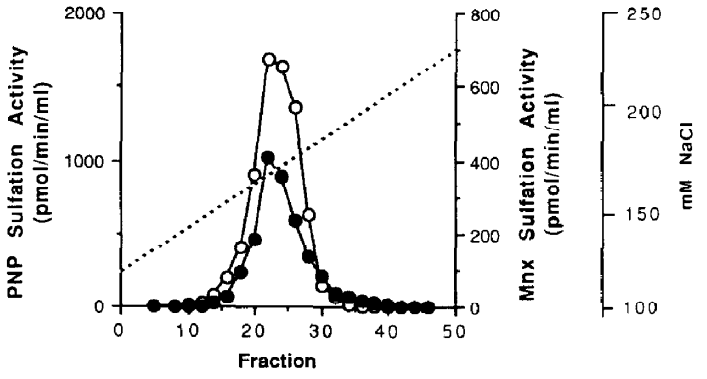

Fig. 3. Elution of Mnx and PNP sulfotransferase activities during DEAE-Sepharose Cl-6B chromatography. Cytosol (600 mg) prepared from HL6 was applied to a DEAE-Sepharose Cl-6B column (2 × 16 cm), and sulfotransferase activities were eluted with a linear 100–225 mM NaCl gradient (600 mL, dashed line) as described in Materials and Methods. Mnx (●) and PNP (○) sulfation activities eluted at a NaCl concentration of approximately 150 mM.

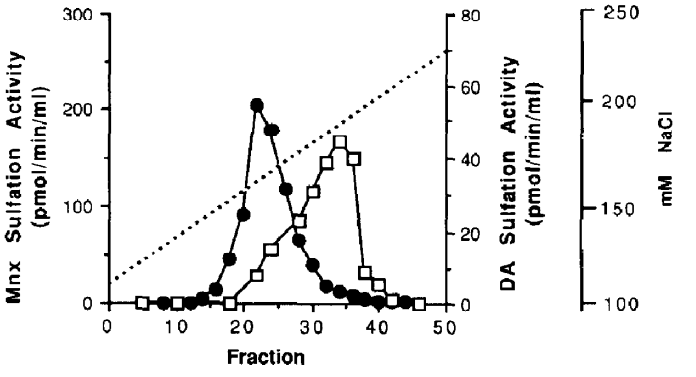

Fig. 4. Elution of Mnx and dopamine sulfotransferase activities during DEAE-Sepharose Cl-6B chromatography. Mnx (●) and dopamine (□) sulfotransferase activities were assayed in the fractions described in Fig. 3. Reaction mixtures contained 10 μM PAPS and either 7 mM Mnx or 10 μM dopamine.

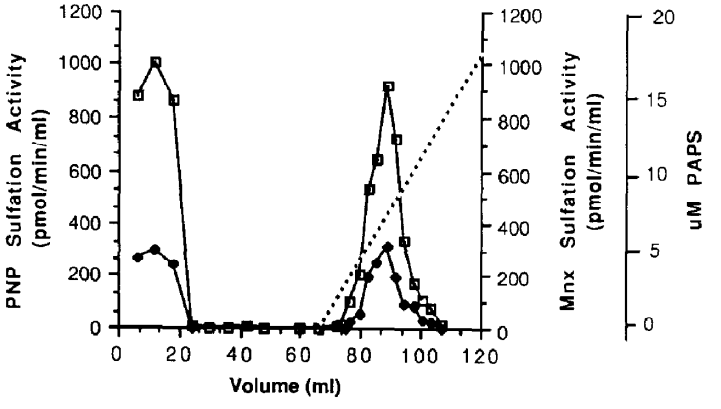

Fig. 5. PAP-agarose affinity chromatography of Mnx and PNP sulfotransferase activities. Fractions from the anion-exchange chromatography procedure described in Fig. 3. were concentrated and applied to a PAP-agarose affinity column (0.7 × 10 cm). PNP (□) and Mnx (◆) sulfotransferase activities were eluted from the affinity column with a linear PAPS gradient (0–20 μM, dashed line) as described in Materials and Methods.

Table 1. Purification of minoxidil (Mnx) and *p*-nitrophenol (PNP) sulfotransferase activities from human liver cytosol

Purification step	Protein (mg)	Total activity (units)* Mnx	Total activity (units)* PNP	Specific activity (units/mg)* Mnx	Specific activity (units/mg)* PNP	Ratio (PNP act./Mnx act)
Cytosol†	924	159	477	0.17	0.52	3.0
DEAE-Sepharose	34	86	246	2.5	7.2	2.9
PAP-agarose	0.38	33	100	87	263	3.0

* One unit equals 1 nmol substrate conjugated/min.
† Cytosol was prepared from liver obtained from a 56-year-old male donor (HL6).

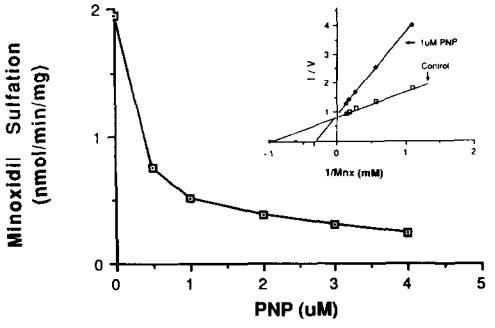

Fig. 6. Inhibition of Mnx sulfation by PNP. Mnx sulfotransferase activity was assayed with 7 mM Mnx and 10 μM PAPS in the presence of increasing amounts of PNP to determine if PNP inhibited Mnx sulfation catalyzed by affinity purified P-PST. The inset is a plot of $1/v$ vs 1/Mnx concentration, showing the effect of various Mnx concentrations in the presence and absence of 1 μM PNP on Mnx sulfotransferase activity.

or DHEA sulfotransferase, the other two sulfotransferases identified in human liver cytosol [12, 13, 16]. Also, the ratio of PNP sulfation to Mnx sulfation was constant during the purification procedure, indicating that an unknown sulfotransferase also present in human liver is probably not involved in sulfating Mnx at detectable levels.

Although P-PST is capable of sulfating the *N*-oxide position of Mnx, other compounds possessing similar functional groups remain to be tested as substrates. Johnson *et al.* [19] have reported previously that rat liver cytosol is capable of sulfating a number of *N*-oxide containing compounds, including Mnx. This result suggests that P-PST may also sulfate other *N*-oxide containing compounds. Also, the ability of P-PST to sulfate the *N*-oxide of Mnx suggests that the enzyme may be capable of sulfating and bioactivating *N*-hydroxyl arylamine compounds, such as *N*-hydroxy-2-acetylaminofluorene, to potent electrophiles [20].

A 14-fold range in the level of Mnx sulfation was observed by Johnson and Baker [8] in platelets obtained from forty-eight male subjects. A 10-fold range in P-PST activity was observed in this report with a small sample of normal livers obtained from both male and female donors [12]. No striking differences were observed in Mnx sulfation between livers obtained from these male and female subjects.

Although Mnx sulfation has been detected in human platelets and liver, it is not known whether the levels of Mnx sulfation in these tissues can be correlated to the efficacy of Mnx in producing its therapeutic effects. However, identification of P-PST as the enzyme responsible for Mnx sulfation will help in investigating the role of inter-individual variations in Mnx sulfation as well as providing a better understanding of the role of sulfation in the bioactivation of therapeutically important drugs.

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