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Activation of 2-Hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine by cDNA-expressed Human and Rat Arylsulfotransferases

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Sulfation plays an obligatory role in the activation of N-hydroxy derivatives of carcinogenic arylamine(amide)s and heterocyclic amines. We found that the hepatic sulfotransferase-mediated covalent binding of ^{3}H-labeled 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (N-OH-PhIP) to calf thymus DNA was 3.3 and 12.9 times higher with human cytosol preparation than with male and female rat cytosol preparations, respectively, in the presence of 3′-phosphoadenosine 5′-phosphosulfate. To assess the activating capacities of individual phenol-sulfating sulfotransferases, five different forms, human ST1A2 and ST1A3 and rat ST1A1, ST1B1 and ST1C1, were expressed in heterologous cells. All five sulfotransferases mediated the activation of N-OH-PhIP to DNA-bound products. The extents of the binding, however, differed considerably among these forms. Human ST1A2 and ST1A3 mediated the activation of N-OH-PhIP at 5.2- and 6.2-fold higher rates than did rat ST1C1, a main N-hydroxy-2-acetylaminofluorene-activating sulfotransferase, in rat liver. Extents of the binding of N-OH-PhIP in human hepatic cytosols of different individuals were positively correlated with the contents of immunoreactive ST1A2/3. These results suggest a potential role of human liver sulfotransferases in N-OH-PhIP activation. In contrast, the low sulfotransferase-mediated activation of N-OH-PhIP in rat liver is consistent with the lack of PhIP hepatocarcinogenicity in this species.

Key words: Pyrolysate carcinogen — DNA binding — Human liver — Sulfation — cDNA expression

Carcinogenic arylamines and arylamides with heterocyclic and carbocyclic moieties require prior metabolic activation to exert their mutagenicity and carcinogenicity. This metabolic activation is accomplished mainly through the N-hydroxylation and successive O-acetylation and O-sulfation of the N-hydroxy derivatives.[1–3] Recently, some knowledge of the primary structures of forms of sulfotransferase has been gained through the use of molecular cloning techniques. Subsequently, a classification of sulfotransferases was proposed based on the primary structures.[4] Hepatic cytosols of rats and humans have been shown to contain enzymes activating N-hydroxy-2-aminofluorene (N-OH-AF), N-hydroxy-2-acetylaminofluorene (N-OH-AAF) and also a food-derived carcinogenic intermediate, 2-hydroxyamino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole (N-OH-Glu-P-1), through a 3′-phosphoadenosine 5′-phosphosulfate (PAPS)-dependent sulfation.[3,5–7] We purified two N-hydroxyarylamine sulfotransferases from rat liver, and showed that these homogeneous proteins catalyzed both N-hydroxyarylamine and *p*-nitrophenol sulfations.[8] Among food-derived carcinogens, 2-amino-1-methyl-6-phenylimidazo-[4,5-*b*]pyridine (PhIP) is one of the most abundant heterocyclic amines by weight in cooked foods, such as fried ground beef and fish.[9] As with other heterocyclic amines, N-hydroxylation is reported to be an initial activation process for PhIP in rat liver.[10] In addition, O-esterification of 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (N-OH-PhIP) is suggested to be necessary for producing the active intermediate capable of binding to DNA.[11] Although cytosolic sulfotransferase mediates the activation of N-OH-PhIP in rats and monkeys, the molecular forms involved in the N-OH-PhIP activation remain unclear. In the present study, we have conducted experiments using cDNA-expression systems to determine the capacity of arylsulfotransferases from human (ST1A2[4] and ST1A3[4]) and rat (ST1A1,[12] ST1B1[4] and ST1C1[13]) for supporting the PAPS-dependent activation of N-OH-PhIP.

MATERIALS AND METHODS

Chemicals PAPS, 3′,5′-adenosine diphosphate, *p*-nitrophenol and *p*-nitrophenyl sulfate were purchased from

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Sigma Chemical Co. (St. Louis, MO). PhIP and 2-amino-4′-hydroxy-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (4′-OH-PhIP) were kindly donated by Dr. Keiji Wakabayashi (National Cancer Center Research Institute, Tokyo). All other chemicals used were of the highest grade available.

Preparation of [^{3}H]N-OH-PhIP [^{3}H]PhIP (1.3 Ci/mmol) was purified by silica gel chromatography prior to use. The nitro derivative of [^{3}H]PhIP was prepared by the method of Turesky *et al.*[14] Briefly, 50 mg of [^{3}H]PhIP (20 mCi/mmol) was added at room temperature over 3-min periods to 5 ml of aqueous sodium nitrite solution (469 mg/ml). After 30 min, the synthesized nitro derivative of PhIP was purified through a Sep-pak C_{18} cartridge. [^{3}H]N-OH-PhIP (20 mCi/mmol) was prepared by the reduction of the nitro derivative of PhIP (20 mCi/mmol) with hydrazine and 10% palladium on activated charcoal as a catalyst. Synthesized [^{3}H]N-OH-PhIP was purified on a Sep-pak C_{18} cartridge. The synthesis of N-OH-PhIP was confirmed by high-performance liquid chromatography/photo-diode array spectral detection methods.[7]

Preparation of hepatic cytosols from rats and humans Hepatic cytosols were prepared from male and female Sprague-Dawley rats (9-week-old) as described previously.[8] Human livers, obtained as surgical samples from the John A. McClellan Memorial Veteran's Hospital, were immediately placed in cold saline, then frozen in liquid nitrogen and stored at −80°C until use. The livers were thawed, washed, and homogenized in 10 m*M* triethanolamine (pH 7.4), 0.25 *M* sucrose and 5 m*M* 2-mercaptoethanol. The homogenates were subjected to sequential centrifugation at 10,000*g* for 10 min, followed by 105,000*g* for 45 min. The supernatants were used as cytosols.

DNA binding assay Metabolic activation of [^{3}H]N-OH-PhIP was estimated by means of a PAPS-dependent DNA binding assay.[15] A reaction mixture (0.5 ml) contained 50 m*M* potassium phosphate (pH 7.5), 5 m*M* $MgCl_2$, 0.5 m*M* ethylenediaminetetraacetic acid (EDTA), 100 μM [^{3}H]N-OH-PhIP, 20 μM PAPS, 5 m*M* *p*-nitrophenyl sulfate, 2 mg/ml calf thymus DNA and 0.7 mg/ml cytosolic fraction. The reaction was initiated by the addition of [^{3}H]N-OH-PhIP, and terminated by the addition of 2 ml of water-saturated *n*-butanol after incubation at 37°C for 30 min. In an experiment with cytosols of human individuals, the amount of liver cytosols added was 2 mg/ml. Metabolic activation of [^{3}H]N-OH-AAF was determined at a concentration of 100 μM in a buffer system of 50 m*M* potassium phosphate (pH 7.5). The reacted mixture was extracted with *n*-butanol three times after addition of 1.5 ml of water, followed by extraction with water-saturated phenol and with chloroform. Modified DNA, recovered by precipitation with 5 ml of chilled ethanol after addition of 50 μl of 5 *M* NaCl, was dissolved in 0.5 ml of water. The DNA was reprecipitated with 3 ml of chilled ethanol:acetone (1:1), and was dissolved in 1.25 ml of water. The radioactivity incorporated in the DNA was determined by liquid scintillation counting, and the amount of DNA was determined according to the method of Burton *et al.*[16] Extents of covalent binding were represented as pmol/mg DNA/mg protein/30 min of incubation and also as pmol/mg DNA/nmol sulfotransferase/30 min of incubation. Measurements were done in triplicate. The data are presented after subtraction of the extent of binding in the absence of PAPS for each substrate.

Expression of arylsulfotransferase cDNAs in COS-1 cells and *Escherichia coli* ST1A1, ST1B1, ST1C1 and ST1A3 cDNAs were each ligated with a mammalian expression vector p91023(B)[17] and then transfected into COS-1 cells by electroporation[18, 19] using a Gene Pulsar apparatus (Bio-Rad Laboratories, Richmond, CA). We isolated two related cDNAs (S-1 and S-2) from two liver cDNA libraries from different human individuals using a rat ST1A1 cDNA as a probe. A possible initiation codon was found in the S-2 cDNA. As compared to S-2 cDNA, S-1 cDNA was judged to lack the first 23 nucleotides from the presumed initiation codon. To supply information on the absent 23 nucleotides, a genomic clone GS-1 corresponding to S-1 cDNA was isolated. The nucleotide sequence of S-1 was identical with that in the putative amino acid coding region of GS-1. Thus, the sequence of the absent 23 nucleotides of S-1 cDNA was taken to be that in GS-1. A protein designated ST1A2[4] was encoded by 885 base pairs of the S-1 including the "absent" nucleotides described above. Another protein designated ST1A3[4] was encoded by 885 base pairs of S-2 cDNA. In order to see whether S-1 cDNA encoded a portion of a functionally active sulfotransferase, S-1 cDNA was ligated with a prokaryotic expression vector pKK233-2 containing the translation initiation codon (ATG) and transfected into *E. coli*.[6] As a result, the N-terminal amino acids of the native ST1A2 (MELIQDISRPP) were altered to MAIPRPP in the *E. coli*-expressed ST1A2. The protein expression was induced by the addition of 1 m*M* isopropylthiogalactopyranoside (IPTG) to the culture medium. The culture was further incubated for 2 h in the presence of IPTG. cDNA-expressed cells were lysed by sonication and the lysate was centrifuged at 105,000*g* for 1 h. The supernatant proteins were analyzed by Western blotting using rabbit antisera against a rat liver arylsulfotransferase.[13, 19]

[6] Details of the structures of the ST1A2 and ST1A3 genes and expression of ST1A2 and ST1A3 will be presented elsewhere.

Metabolic labeling and immunoprecipitation of sulfotransferases expressed in COS-1 cells Eukaryotic expression vector p91023(B) (100 μg) carrying each sulfotransferase cDNA was transfected into COS-1 cells (2×10^7 cells) by electroporation.[17-19] The transfected cells were incubated in 10 ml of minimum essential medium supplemented with 10% dialyzed fetal calf serum and 100 μCi of [^{35}S]methionine (NEN Research Products) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C for 72 h. The cells were washed with phosphate-buffered saline three times and lyzed in 5 ml of solubilization buffer (Buffer A) containing 10 m*M* Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.15 *M* NaCl, 1 m*M* EDTA and 10 μg/ml aprotinin. Rabbit antisera (50 μl) against a rat arylsulfotransferase were added to the cell lysates pretreated with 25 μl of protein A-Sepharose (50% (v/v) suspension, Pharmacia). Then antibody-recognized sulfotransferases were precipitated by the addition of 40 μl of protein A-Sepharose after a 60-min incubation on ice. Precipitates were washed five times with 5 ml of Buffer A, resuspended in 640 μl of buffer containing 10% glycerol, 5% 2-mercaptoethanol, 6% SDS and 130 m*M* Tris-HCl (pH 6.8), and heated at 100°C for 2 min. After a brief centrifugation, proteins in the supernatant (80 μl) were separated on SDS-polyacrylamide gel electrophoresis and blotted onto a nitrocellulose sheet. Metabolically ^{35}S-labeled sulfotransferases were probed with the same anti-arylsulfotransferase antibodies as used for immunoprecipitation and visualized by diaminobenzidine staining. After diaminobenzidine staining, the nitrocellulose sheet was exposed to an imaging plate using a Bioimaging Analyzer BAS2000 (Fujix, Tokyo) to detect metabolically ^{35}S-labeled and immunoprecipitated sulfotransferases. Radioactivity of each labeled sulfotransferase was quantified from the intensity on the screen. The numbers of methionine residues per subunit (deduced from nucleotide sequences) were 9, 9, 15 and 13 residues in ST1A1,[12] ST1B1,[4] ST1C1[13] and ST1A3,[4] respectively. Relative molar ratio (ST1A1:ST1B1:ST1C1:ST1A3) of metabolically labeled sulfotransferases expressed in COS-1 cells were calculated as 0.29:0.27:1:3.75 from the radioactivity and the number of methionine residues per subunit. Based on a comparison of the protein band intensity of ^{35}S-labeled ST1C1 and ST1C1 purified from rat liver (HAST protein[8]), the absolute amounts of the ^{35}S-labeled sulfotransferases were determined. Expressed levels of sulfotransferases in cells used for measurement of N-OH-PhIP binding were determined by comparison of the immunointensity with that of the ^{35}S-labeled sulfotransferases: 0.037 nmol/mg COS-1 cell cytosol for ST1A1; 0.034 for ST1B1; 0.143 for ST1C1; 0.110 for ST1A3. During the immunoprecipitation procedure, about 3% of total sulfotransferase expressed in cells was recovered and detected on a nitrocellulose sheet.

Assay of sulfotransferase *p*-Nitrophenol sulfotransferase activity was measured as described previously.[13,19,20] The reaction mixture (10 μl) for human sulfotransferases contained 50 m*M* potassium phosphate buffer (pH 6.8), 5 m*M* magnesium chloride, 1 m*M* dithiothreitol (DTT), 4 μ*M* *p*-nitrophenol, 200 μ*M* [^{35}S]PAPS and 0.1 mg/ml cytosolic fraction. The reaction mixture (250 μl) for dopamine sulfotransferase assay[19,21] for human sulfotransferases consisted of 50 m*M* potassium phosphate (pH 6.8), 5 m*M* $MgCl_2$, 50 μ*M* dopamine, 1 m*M* pargyline, 10 μ*M* [^{35}S]PAPS and 0.1 mg/ml cytosolic protein. For the measurement of dopamine sulfation by rat sulfotransferases, 50 m*M* glycine-NaOH (pH 9.0) was used[19,21] instead of 50 m*M* potassium phosphate (pH 6.8). Minoxidil sulfotransferase activity was determined as described by Johnson and Baker.[22] β-Naphthol sulfotransferase activity was measured using *p*-nitrophenylsulfate as a sulfate donor according to the method by Mulder *et al.*[23] with some modifications. The incubation mixture contained 50 m*M* potassium phosphate buffer (pH 6.3), 5 m*M* magnesium chloride, 5 m*M* *p*-nitrophenyl sulfate, 20 μ*M* 3′,5′-adenosine diphosphate, 100 μ*M* β-naphthol and 0.6 mg/ml cytosolic fractions. The activity was calculated based on the increase in A_{405} due to release of *p*-nitrophenol. O-Sulfation of 4′-OH-PhIP (0.5 m*M*) and N-sulfation (sulfamate formation) of PhIP (1 m*M*) were examined in the presence of cytosols of cells expressing rat ST1A1 and ST1C1 and rat livers. Each of the reaction products, PhIP 4′-sulfate and PhIP-sulfamate, was visualized on cellulose thin layer chromatograms.[20]

RESULTS

Sulfotransferase-mediated activation of N-OH-PhIP in human and rat livers Significant binding of N-OH-PhIP to calf thymus DNA occurred in the presence of PAPS as a cofactor in systems containing liver cytosols of humans and rats. As can be seen in Table I, the PAPS-dependent binding of N-OH-PhIP in human liver cytosols varied considerably among individuals (88.5±40.0 pmol/mg DNA/mg cytosolic protein/30 min). The mean level was 3.3- and 12.9-fold higher than those in liver cytosols of male and female rats, respectively. This is in clear contrast to N-OH-AAF binding in human and rat liver cytosols. N-OH-AAF was very efficiently activated in liver cytosols of male rats. The N-OH-AAF binding in liver cytosols of the male rats (1670±90 pmol/mg DNA/mg cytosolic protein/30 min) was 31.3-fold higher than that in liver cytosols of humans. The binding in hepatic cytosols of the female rats (103.1±54.9 pmol/mg DNA/mg cytosolic protein/30 min) was much less

Table I. Sulfotransferase-mediated Activation of N-OH-PhIP and N-OH-AAF in Human and Rat Liver Cytosols[a)]

	Human	Rat	
		Male	Female
	(pmol/mg DNA/mg cytosolic protein/30 min)[b)]		
N-OH-PhIP	88.5±40.0	27.1±2.2	6.9±3.1
N-OH-AAF	53.3±23.7	1670±90	103.1±54.9

a) Sulfotransferase-mediated covalent binding of N-OH-PhIP to calf thymus DNA was measured in a system containing 100 μM [^{3}H]N-OH-PhIP, 2 mg/ml calf thymus DNA, 20 μM PAPS and 0.7 mg/ml liver cytosols of humans (n=12) or rats (n=3 for each sex) in a total volume of 0.5 ml. DNA was purified by phenol extraction and ethanol precipitation as described in "Materials and Methods." Rates of DNA binding shown are after subtraction of the values in the absence of PAPS.
b) Mean±SD.

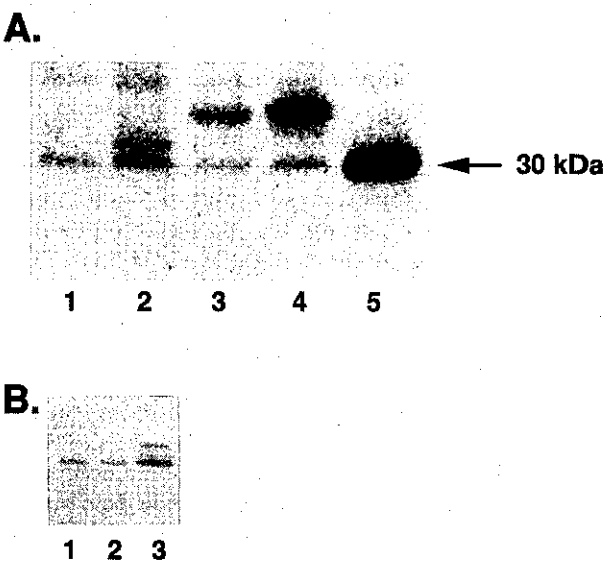

Fig. 1. A. Western blots of [^{35}S]methionine-labeled sulfotransferases immunoprecipitated from cDNA-expressed COS-1 cells. Immunoreactive sulfotransferases were precipitated from COS-1 cells expressing ST1A1, ST1B1, ST1C1 and ST1A3 as described in "Materials and Methods." The immunoprecipitated and ^{35}S-labeled sulfotransferases, which were subjected to Western blotting, were visualized by measuring the radioactivity with a Bioimaging Analyzer BAS2000. Lane 1, null construct-transfected cells; lane 2, ST1B1 cDNA-expressed cells; lane 3, ST1A1 cDNA-expressed cells; lane 4, ST1C1 cDNA-expressed cells; lane 5, ST1A3 cDNA-expressed cells. B. Immunoblots of cytosols of ST1A2-expressing *E. coli*, ST1A3-expressing COS-1 cells and a human liver cytosol. Cytosols (70 μg) from sulfotransferase-expressing cells and human livers were separated by SDS-polyacrylamide gel electrophoresis (10.5%), electroblotted onto a nitrocellulose membrane, and probed with rabbit anti-rat arylsulfotransferase antibodies. The immunodetectable sulfotransferases were visualized by diaminobenzidine staining. Lane 1, cytosol from *E. coli* expressing ST1A2; lane 2, cytosol from COS-1 cells expressing ST1A3; lane 3, human liver cytosol.

than that of male rats, but still 1.9-fold higher than that of humans. The sex-related difference is consistent with our previous results.[24)] These findings suggest a difference in substrate specificities among the forms of mammalian cytosolic sulfotransferases.

Metabolic labeling and immunoprecipitation of sulfotransferases expressed in COS-1 cells Before the comparison of the capacity of sulfotransferase forms to activate N-OH-PhIP, the amounts of sulfotransferase expressed in COS-1 cells were quantitated using [^{35}S]methionine. Human and rat sulfotransferases labeled with [^{35}S]methionine were precipitated by rabbit anti-arylsulfotransferase antibodies. The complex was separated with protein A-Sepharose, and subjected to Western blotting to determine the location of sulfotransferases immunochemically (data not shown) and also by measuring the radioactivity with a Bioimaging Analyzer BAS2000 (Fig. 1A). Under the current experimental conditions, a faint band of immunoprecipitated protein was detected at around 30 kDa in null construct-transfected cells. ST1A1, ST1B1, ST1C1 in COS-1 cells were separable from this band and appeared at 32.5 kDa, 31 kDa and 33 kDa, respectively, in accordance with our previous results.[4, 13, 19)] ST1A3 migrated at the same position as the unknown protein band, whose radioactivity was subtracted to estimate the expressed level of ST1A3. Contents of sulfotransferases expressed in COS-1 cells were determined as described in "Materials and Methods." Considerable differences were observed in the expressed levels of the arylsulfotransferases: 0.037 nmol/mg COS-1 cell cytosol for ST1A1, 0.034 for ST1B1, 0.143 for ST1C1 and 0.110 for ST1A3. As shown in Fig. 1B, ST1A2 and ST1A3 were migrated to the same position as a major protein band (30 kDa) observed in human livers. The ratio of immunodetectable amounts of ST1A2 and ST1A3 expressed in *E. coli* and COS-1 cells was 1.10:1.00 (ST1A2:ST1A3) as judged from Western blots using rat arylsulfotransferase antibodies. ST1A2 and ST1A3 are structurally related, and their deduced amino acid sequences differed in only 12 residues out of 295 after alignment. In the present study, the amino acid sequences of expressed ST1A2 and ST1A3 showed 94% similarity after the modification of N-terminal amino acids in ST1A2 as described in "Materials and Methods." Cytosolic content of ST1A2 was estimated as 0.121 nmol/mg *E. coli* cell cytosol, assuming that the anti-sulfotransferase antibody reacts equally with ST1A2 and ST1A3.

Sulfotransferase activities in cDNA-expressed cells Activities of cDNA-expressed sulfotransferases were examined toward typical arylsulfotransferase substrates, *p*-nitrophenol, dopamine and minoxidil, in the presence of PAPS as a cofactor. For β-naphthol sulfation, *p*-nitrophenyl sulfate was used as a sulfate donor. Although all the sulfotransferases examined catalyzed sulfations of *p*-nitrophenol and minoxidil, the specific activities of the enzymes were clearly different (Table II). Human ST1A3 catalyzed sulfations of *p*-nitrophenol, minoxidil and β-naphthol at rates 97-, 14- and 21-fold higher than did ST1A2, respectively. ST1A2 and ST1A3 had no appreciable activity toward dopamine under the experimental conditions used. Rat ST1A1 showed broader substrate specificity than the other rat enzymes. ST1B1 showed the highest activity for *p*-nitrophenol sulfation among all the forms of sulfotransferase examined, but had no appreciable activity toward β-naphthol. ST1C1 also catalyzed *p*-nitrophenol sulfation at a rate 1.8-fold higher than did ST1A1, but had low minoxidil-sulfating activity (3%) as compared to ST1A1.

Table II. Sulfotransferase Activities of ST1A2, ST1A3, ST1A1, ST1B1 and ST1C1[a)]

Substrate	ST1A2	ST1A3	ST1A1	ST1B1	ST1C1
	(pmol/min/nmol sulfotransferase)				
p-Nitrophenol	30	2920	3600	9770	6640
Dopamine	<20	<20	530	60	330
Minoxidil	40	540	3320	3380	110
β-Naphthol	1700	35500	40500	<1500	18900

a) Sulfotransferase activities were measured at the concentrations of 4 μ*M* at pH 6.8 (ST1A2 and ST1A3) or 1 m*M* at pH 5.5 (ST1A1, ST1B1 and ST1C1) for *p*-nitrophenol, 50 μ*M* at pH 6.8 (ST1A2 and ST1A3) or 50 μ*M* at pH 9.0 (ST1A1, ST1B1 and ST1C1) for dopamine, 1 m*M* for minoxidil and 100 μ*M* for β-naphthol in duplicate determinations. The experimental conditions for each of the rat and human enzymes were optimum. Deviations were within 7% of the mean. 3′-Phosphoadenosine 5′-phosphosulfate was used as a sulfate donor except for β-naphthol. *p*-Nitrophenyl sulfate was used as a sulfate donor for β-naphthol. Activities in the cells transfected with the null construct were subtracted from those in the cells expressing each sulfotransferase. Sulfotransferase activities were expressed as pmol/min/nmol sulfotransferase, taking the expressed level of each sulfotransferase into consideration.

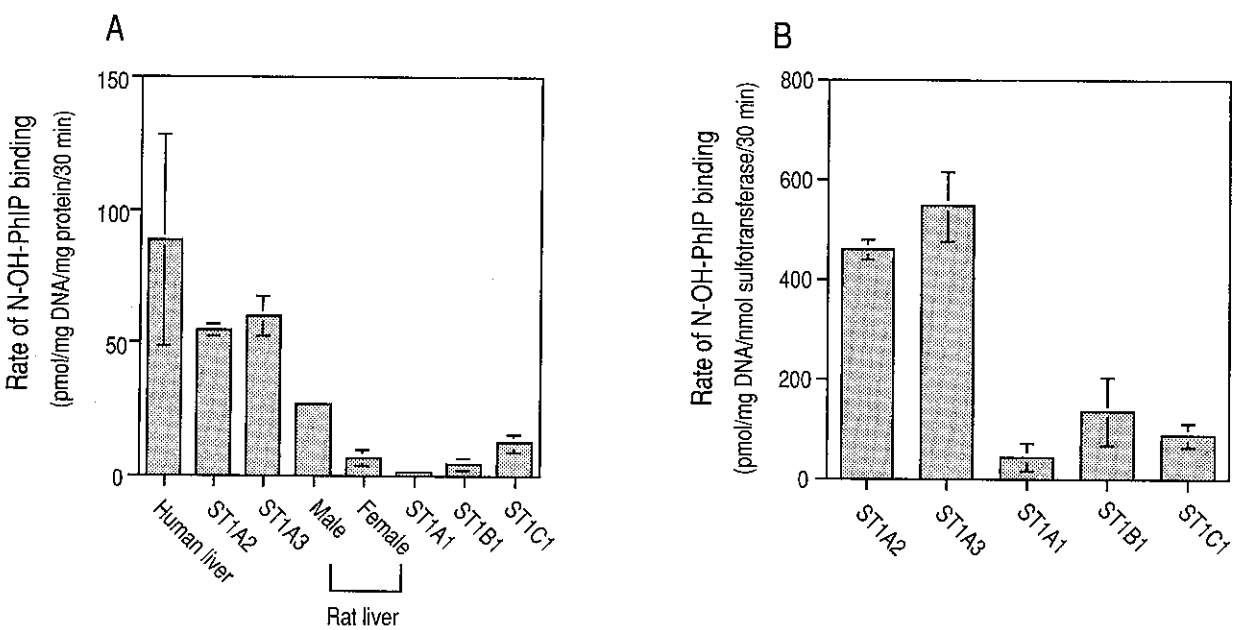

Fig. 2. PAPS-dependent DNA binding of [^{3}H]N-OH-PhIP. [^{3}H]N-OH-PhIP was incubated with 2 mg/ml calf thymus DNA at the concentration of 100 μ*M* in the presence of 20 μ*M* PAPS, and 0.7 mg/ml cytosol from cDNA-expressed cells or human livers in a total volume of 0.5 ml at 37°C for 30 min. DNA was purified by phenol extraction and ethanol precipitation as described in "Materials and Methods." The radioactivity was measured by liquid scintillation counting. Rates of DNA binding in the cDNA-expressed cells are shown in A after subtraction of the values in the cells transfected with the null construct. Data on DNA binding in the cDNA-expressed cells shown in A were converted to rates of DNA binding per nmol sulfotransferase expressed in cells (B).

DNA bindings of N-OH-PhIP catalyzed by cDNA-expressed sulfotransferases Significant binding of N-OH-PhIP to calf thymus DNA occurred in systems containing cytosols of cDNA-expressed cells, and liver cytosols of rats and humans (Fig. 2A). The data on DNA binding are shown as pmol/mg DNA/nmol sulfotransferase/30 min, taking the expressed level of each sulfotransferase into consideration. As shown in Fig. 2B, human ST1A2 and ST1A3 mediated covalent binding of N-OH-PhIP to DNA at rates of 457.1±19.0 pmol/mg DNA/nmol ST1A2/30 min and 545.5±70.1 pmol/mg DNA/nmol ST1A3/30 min, respectively. Covalent binding of N-OH-PhIP catalyzed by rat ST1A1 (41.2±29.1 pmol/mg DNA/nmol ST1A1/30 min) amounted to no more than 9% and 8% of those catalyzed by ST1A2 and ST1A3, respectively. Rat ST1B1 and ST1C1 also catalyzed activation of N-OH-PhIP, but exhibited weak N-OH-PhIP binding as compared to human ST1A3 (25% and 16%, respectively). These results are coincident with the higher capacity of human liver cytosols to activate N-OH-PhIP in comparison with rat liver cytosols (Table I).

Correlation between levels of immunodetectable 30 kDa protein and N-OH-PhIP binding Considerable individual differences are observed in the hepatic phenol-sulfating activities of human livers. Human ST1A2 and ST1A3, which catalyzed *p*-nitrophenol sulfation (Table I), were detected at 30 kDa in a Western blot using anti-rat arylsulfotransferase antibody (Fig. 1B). A major immunodetectable protein in human liver also migrated to a position corresponding to 30 kDa, as did ST1A2 and ST1A3 (Fig. 1B). Therefore, the correlations of extent of N-OH-PhIP binding with *p*-nitrophenol sulfation and total content of ST1A2/3 were assessed. Extent of N-OH-PhIP binding was well correlated with *p*-nitrophenol sulfation (Fig. 3A, $r=0.910$) and also with total content of ST1A2/3 (Fig. 3B, $r=0.738$).

O-Sulfation of 4′-OH-PhIP and sulfamate formation of PhIP in cytosols of cells expressing ST1A1 and ST1C1 and rat livers PhIP was shown to be hydroxylated at the 4′-position and further O-sulfated to yield PhIP 4′-sulfate, a major detoxication reaction of PhIP.[25] Studies on the *in vivo* metabolism of 2-amino-3-methylimidazo-[4,5-*f*]quinoline (IQ) showed that IQ was detoxified and

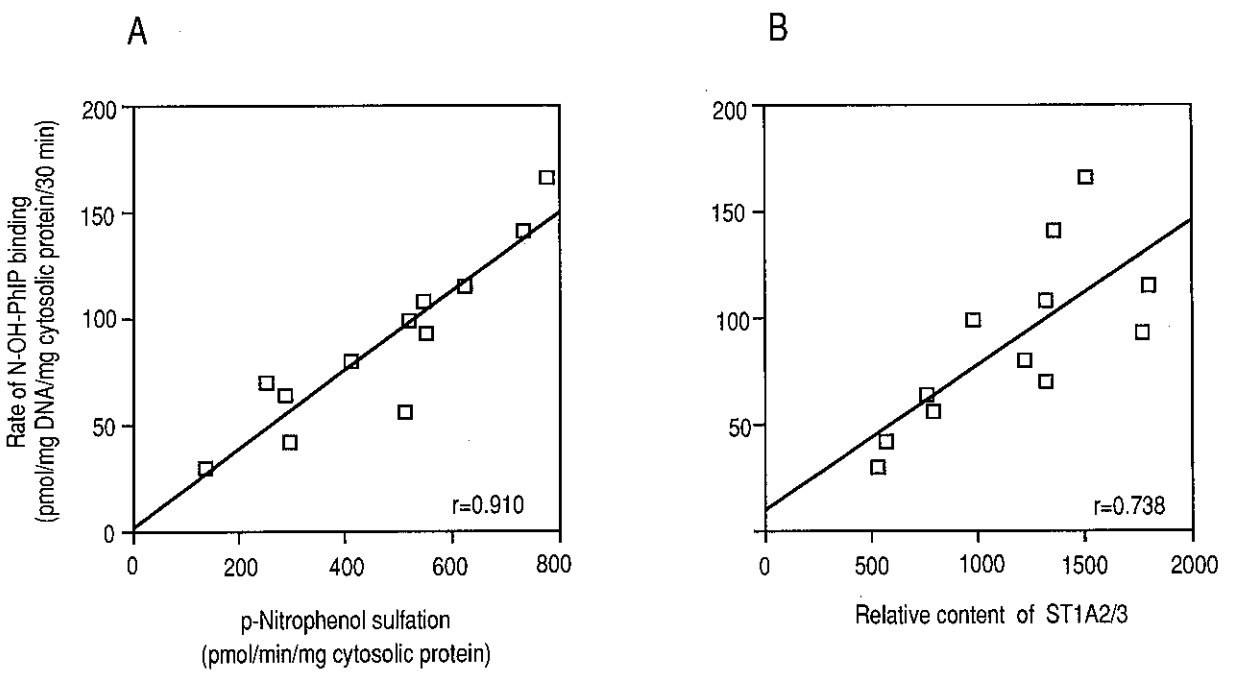

Fig. 3. Correlation between *p*-nitrophenol sulfation (A) or relative hepatic content of ST1A2/3 (B) and rate of N-OH-PhIP binding to calf thymus DNA. N-OH-PhIP binding catalyzed human liver cytosols (n=12) was measured at the concentration of 100 μM as described in "Materials and Methods." *p*-Nitrophenol sulfation was determined as the concentration of 4 μM. Cytosols (70 μg) of human livers were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10.5%), electroblotted onto a nitrocellulose membrane, and probed with rabbit anti-rat arylsulfotransferase antibodies. Immunoreactive ST1A2/3 proteins (30 kDa) were quantified by densitometric scanning.

Table III. O-Sulfation of 4′-OH-PhIP and Formation of PhIP-sulfamate in Cytosols of Sulfotransferase-expressing COS-1 Cells and Rat Livers

Substrate	COS-1 cells		Rat livers	
	ST1A1	ST1C1	Male	Female
4′-OH-PhIP	7 (189)	252 (1762)	148±7	74±10
PhIP	2 (54)	1 (7)	14±1	30±5

O-Sulfation of 2-amino-4′-hydroxyl-1-methyl-6-phenylimidazo-[4,5-*b*]pyridine (4′-OH-PhIP, 0.5 m*M*) and sulfamate formation of PhIP (1 m*M*) was measured in the system containing 10 m*M* potassium phosphate (pH 7.2), cytosol (0.3–0.4 mg/ml) of sulfotransferase-expressing cells or rat livers, and 25 μM [^{35}S]PAPS. Activities are expressed as pmol/min/mg cytosolic protein and also as pmol/min/nmol sulfotransferase (in parentheses), taking the expressed level of ST1A1 and ST1C1 in cells (0.037 and 0.143 nmol/mg cytosolic protein, respectively) into consideration.

excreted to a considerable extent as a sulfamate.[26] We examined O-sulfation of 4′-OH-PhIP and sulfamate formation of PhIP using 25 μM [^{35}S]PAPS as a cofactor. As can be seen in Table III, both PhIP 4′-sulfate and PhIP-sulfamate were formed in the presence of cytosols of ST1A1- and ST1C1-expressing cells and rat livers. O-Sulfations of 4′-OH-PhIP catalyzed by liver cytosols of male and female rats amounted to 148 and 74 pmol/min/mg cytosolic protein, respectively, which were 10.5- and 2.5-fold higher than PhIP sulfamate formation catalyzed by liver cytosols of male and female rats. Consistently, ST1A1- and ST1C1-catalyzed formations of PhIP 4′-sulfate were 3.5- and 250-fold greater than PhIP-sulfamate formation, respectively. These results suggested that forms of cytosolic sulfotransferase are involved in various metabolic pathways of PhIP.

DISCUSSION

In regard to sulfotransferase-mediated activation of N-OH-PhIP, tissue cytosols of primates have been shown to mediate higher levels of DNA binding of N-OH-PhIP than tissue cytosols of male rats.[11] This is in clear contrast to the activation of N-OH-AAF and N-OH-Glu-P-1; the extents of the PAPS-dependent binding of these N-hydroxy derivatives in male rat livers were at least 5-fold higher than those in human livers.[24] Consistently, covalent binding of N-OH-PhIP to calf thymus DNA in human liver cytosols was 3.3-fold higher than that in rat liver cytosols (Table I). We have determined the capacities of 5 different human and rat aryl(phenol)sulfotransferases to mediate the PAPS-dependent binding of N-OH-PhIP to DNA. The sulfotransferase cDNAs were isolated and expressed in COS-1 cells and *E. coli*. Human ST1A2 and ST1A3 mediated N-OH-PhIP binding at rates of 457.1 and 545.5 nmol/mg DNA/nmol sulfotransferase/30 min, respectively (Fig. 2B). The extents of binding were 3.4- and 4.1-fold higher than that mediated by rat ST1B1, although the latter was the most active among the three rat forms (Fig. 2B). The higher N-OH-PhIP-activating capacity of human ST1A2 and ST1A3 is consistent with the higher N-OH-PhIP binding capacity in human liver cytosols as compared with rat liver cytosols (Table I, Ref. 8). In contrast to N-OH-PhIP, N-OH-AAF binding mediated by rat ST1C1 (15,000 pmol/mg DNA/nmol ST1C1/30 min) was 160- and 39-fold higher than those mediated by human ST1A2 and ST1A3, respectively (data not shown). These results were concordant with the 31.3-fold higher capacity of liver cytosols of male rats to activate N-OH-AAF as compared with liver cytosols of humans (Table I).

Considerable differences are observed in individual hepatic phenol-sulfating activities, which are catalyzed by arylsulfotransferases. Both *p*-nitrophenol sulfation and total content of ST1A2/3 correlated well with extent of N-OH-PhIP binding (Fig. 3A, $r=0.910$ and Fig. 3B, 0.738, respectively) among twelve human individuals. These results are consistent with the fact that ST1A2 and ST1A3 mediated both *p*-nitrophenol sulfation (Table II) and covalent binding of N-OH-PhIP (Fig. 2).

N-OH-PhIP binding was observed at a rate of 27.1 pmol/mg DNA/mg cytosolic protein/30 min in hepatic cytosols of male rats, and this was 3.9-fold higher than that in liver cytosols of female rats. Rat ST1A1, ST1B1 and ST1C1 catalyzed DNA binding of N-OH-PhIP at rates of 41.2, 134.5 and 87.9 pmol/mg DNA/nmol sulfotransferase/30 min, respectively. Similar levels (0.1 nmol/mg cytosol) of ST1A1 were contained in male and female rat livers. ST1C1, whose hepatic content in male rats was about 0.1 nmol/mg cytosol, was expressed in a male-dominant manner. These data suggest that the sex-related difference in N-OH-PhIP binding was caused by the male-specific expression of ST1C1.

In Western blots, very low levels of immunoreactive ST1B1 were detected in rat liver cytosols of both sexes of rats. Data on immunoprecipitation of ST1B1 and on immunoblot analysis of rat liver cytosols suggested that ST1B1 contents in male and female rat liver cytosols were less than 0.02 nmol/mg cytosol (data not shown). These results suggest, at best, a minor contribution of ST1B1 to the N-OH-PhIP activation in rat liver.

PhIP has been found to be carcinogenic to colon and mammary gland, but not to liver of Fischer 344 rats.[27] Higher capacity of ST1A2 and ST1A3 to activate N-OH-PhIP as compared with the rat forms may imply a potential role of hepatic ST1A2/3 in PhIP carcinogenicity in humans, in contrast to the lack of liver tumor induction by PhIP in rats.[27]

In the present study, we have characterized human ST1A2 and ST1A3, and rat ST1A1 and ST1C1 as forms of arylsulfotransferase which mediate N-OH-PhIP activation in the respective animal species. Phenol-sulfating forms of sulfotransferase are expressed in human extrahepatic tissues such as brain,[28] platelets[29] and intestine.[30] ST1A2 and ST1A3 cDNAs may be useful tools to examine tissue specificity in the expression of the N-OH-PhIP-activating enzymes.

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