Down-regulation of sulfotransferase expression and activity
in diseased human livers

Emine B. Yalcin, Vijay More, Karissa Neira, Zhenqiang James Lu,
Nathan J. Cherrington, Angela L. Slitt, and Roberta S. King

Department of Biomedical and Pharmaceutical Sciences, College of Pharmacy,
University of Rhode Island, Kingston, RI (EBY, VM, KN, ALS, RSK)
Arizona Statistical Consulting Laboratory, The Bio5 Institute, The University of Arizona,
Tucson, AZ (ZJL)
Department of Pharmacology and Toxicology, College of Pharmacy, University of
Arizona, Tucson, AZ (NJC)
Running title: Sulfotransferase expression and activity in liver disease

Corresponding Author:

Roberta S. King, Ph.D.

Department of Biomedical and Pharmaceutical Sciences

College of Pharmacy

Pharmacy Building

7 Greenhouse Road, Kingston, RI 02881

401-874-7061 (phone), 401-874-5787 (fax)

Email: rking@uri.edu

Number of text pages: 22

Number of tables: 1

Number of figures: 6

Number of references: 60

Word counts:

Abstract: 201

Introduction: 762

Discussion: 1477

Abbreviations: dehydroepiandrosterone (DHEA), dichlorophenolindophenol (DCPIP), nicotinamide adenine dinucleotide phosphate (NADPH), NAD(P)H:quinone oxidoreductase 1 (NQO1), nonalcoholic fatty liver disease (NAFLD), nonalcoholic steatohepatitis (NASH), para-Nitrophenol (pNP), 3'-Phosphoadenosine-5'-phosphosulfate (PAPS).
Abstract

Sulfotransferase function has been well studied in healthy human subjects by quantifying mRNA and protein expression and determining enzyme activity with probe substrates. However, it is not well known if sulfotransferase activity changes in metabolic and liver disease such as diabetes, steatosis, or cirrhosis. Sulfotransferases have significant roles in the regulation of hormones and excretion of xenobiotics. In the present study, SULT1A1, SULT2A1, SULT1E1, and SULT1A3 activity, mRNA and protein expression in human liver tissue from groups of patients diagnosed with steatosis, diabetic cirrhosis, alcoholic cirrhosis, and normal subjects characterized with non-fatty livers was determined. In general, sulfotransferase activity decreased significantly with severity of liver disease from steatosis to cirrhosis. Specifically, SULT1A1 and SULT1A3 activities were lower in disease states relative to non-fatty tissues. Alcoholic cirrhotic tissues further contained lower SULT1A1 and 1A3 activities than either of the two other disease states. SULT2A1, on the other hand, was only reduced in alcoholic cirrhotic tissues. SULT1E1 was reduced in both diabetic cirrhosis and alcoholic cirrhosis tissues, relative to non-fatty liver tissues. In conclusion, the reduced levels of sulfotransferase expression and activity in diseased versus non-diseased liver tissue may alter the metabolism and disposition of xenobiotics and affect homeostasis of endobiotic sulfotransferase substrates.
Introduction

Sulfonation has a significant role in the biotransformation of numerous endogenous low-molecular weight compounds including catecholamine neurotransmitters, steroids, and thyroid hormones (Negishi et al., 2001). Furthermore, it is an important pathway in the biotransformation of a number of xenobiotics including drugs and chemicals (Negishi et al., 2001). Sulfonation reaction mechanism is based on the substitution of a hydrogen atom from the functional phenolic or alcoholic group of acceptor molecule with a sulfonyl group (\(-\text{SO}_3^-\)) from the universal donor molecule PAPS (3'-phosphoadenosine-5'-phosphosulfate). Thus, the substrate of sulfonation is a neutral phenol or alcohol (R-OH), and the product of sulfonation is a highly polar sulfate (R-\text{OSO}_3^-) at physiological pH (Klaassen and Boles, 1997).

One function of sulfonation is the defense mechanism against certain chemicals via elimination from the body. SULT1A1 is the isoform responsible for the metabolism and subsequent disposition of a number of exogenous substances possessing a small phenolic structure such as acetaminophen (Reiter and Weinshilboum, 1982), 4-hydroxytamoxifen (Falany et al., 2006) and synthetic estrogens (bisphenol A, diethylstilbestrol (DES)) (Suiko et al., 2000). A second function of sulfonation is the inactivation of steroid hormones and neurotransmitters. The sulfated form of estradiol and testosterone are not receptor-active, and sulfonation by SULT2A1 and SULT1E1 serves to reduce concentration of active ligand (Falany, 1997). A third function of sulfonation involves biosynthesis of the androgen and estrogen hormones by enabling their precursor, dehydroepiandrosterone (DHEA), to be transported in plasma in the
soluble form DHEA-sulfate. DHEA-sulfate can be synthesized directly from cholesterol-sulfate or formed from DHEA by SULT2A1 (Falany, 1997).

The presence of sulfated metabolites of serotonin, norepinephrine, and pregnenolone in brain and plasma points out the role of sulfotransferases in neurotransmitter metabolism (Costa et al., 1983; Strobel et al., 1999; Schumacher et al., 2008). In addition, dopamine sulfate is detected at much higher levels in plasma than free dopamine. Free dopamine plasma concentration (< 0.1 pmol/mL) is less than 1% of its sulfate-conjugated form concentration (Eisenhofer et al., 1999). SULT1A3 is responsible for dopamine sulfonation and is highly expressed in the gastrointestinal tract of humans, where the majority of dopamine sulfate (75%) is produced in the body. In animal models dopamine sulfate regulates gut motility in mice (Haskel and Hanani, 1994), intestinal sodium absorption in weaning rats (Finkel et al., 1994), and gastroprotective effects in rats (Glavin, 1991). Its function in human is less well-known.

Type II diabetes mellitus is defined as decreased insulin secretion or insulin sensitivity resulting in elevated fasting blood glucose (Chiang et al., 2011), which composes a risk factor for nonalcoholic fatty liver disease (NAFLD) (Lattuada et al., 2011). NAFLD is the most common chronic liver disease in the industrialized countries. Current estimates of NAFLD range from 5% to 33% of US population, but true prevalence is likely to be higher as many people remain undiagnosed in early stages due to the lack of inexpensive and non-invasive screening tests (Lazo and Clark, 2008; Moore, 2010). NAFLD is characterized as the accumulation of fat in liver in the absence of excessive
alcohol intake. The main pathogenic mechanism of NAFLD is insulin resistance, which can be caused by genetic determinants, poor nutrition, and lifestyle (Pascale et al., 2010). NAFLD ranges from simple steatosis (fat without inflammation) to cirrhosis (McClain et al., 2004). Although development of cirrhosis subsequent to steatosis is becoming much more common, alcohol abuse and viral hepatitis currently remain the most common causes of cirrhosis (Starr and Raines, 2011). 90% of individuals who drink more than 60 g/day of alcohol develop steatosis (Crabb, 1999). This is a benign, asymptomatic condition, usually reversed within 4-6 weeks via the absence of alcohol, with a low risk (5-15%) of developing fibrosis and cirrhosis (Leevy, 1962; Sorensen et al, 1984). However, the risk of progression into cirrhosis increases to 37% with continuous alcohol intake (Teli et al., 1995).

The above mentioned diseases have been found to alter the expression and activity of various enzymes important to drug disposition and homeostasis of endogenous molecules such as steroid hormones, cholesterol, bile acids and neurotransmitters (Merrell and Cherrington, 2011; Buechler and Weiss, 2011). While sulfotransferase is an important regulator of the disposition of these endogenous and exogenous molecules, sulfotransferase activity has not been well-characterized under disease conditions. The findings from this study are expected to clarify the liver tissue expression and activity levels of a major drug metabolism enzyme family at various liver disease states. Our results may help to predict therapeutic dosing and avoid toxicity problems of the drugs that are metabolized via sulfonation, and to better understand the effect of liver disease on homeostasis of endobiotic sulfotransferase substrates.
Materials and Methods

Materials and Chemicals

[35S]PAPS (1.5–2.54 Ci/mmol), [3H]17β-estradiol (110 Ci/mmol), and scintillation fluid (Optima Flo-M) were purchased from PerkinElmer Life and Analytical Sciences. Anti-SULT1A1 (ARP49134_P050) and anti-SULT1E1 (ARP48669_P050) were purchased from Aviva Systems Biology. Anti-SULT1A3 (ab92476) and anti-SULT2A1 (ab38416) were purchased from Abcam. p-Nitrophenol, dopamine, 2,6-dichlorophenolindophenol (DCPIP), dicumarol, and NAD(P)H were purchased from Sigma-Aldrich. Dehydroepiandrosterone was purchased from Steraloids Inc.

Cytosolic fraction from isolated liver tissue

Human liver tissues were purchased from Liver Tissue Cell Distribution System (LTCDS), University of Minnesota, Minneapolis, MN. The details of the human liver donors are described (Supplemental Table 1), according to the data provided by LTCDS: steatosis (n=13 individuals), diabetes (n=4), diabetic cirrhosis (n=22), alcohol cirrhosis (n=22) and non-fatty liver (n=20). Liver samples were stored frozen at -80°C until the cytosolic fractions were prepared. Approximately 300 mg liver tissue of each sample was homogenized in buffer containing 0.25 M sucrose, 1 M Tris HCl (pH 7.8) 0.01 M EDTA, 0.5 mM BHT and 0.1 M DTT with PowerGen 125 tissue homogenizer three times for ~10-20 seconds keeping the tissue on ice in between. The homogenate was ultra-centrifuged at 100,000 x g for one hour, 4°C with Sorvall Discovery M120 SE, Hitachi. Aliquots of supernatant (cytosol) were stored at -80°C until assay. The protein
concentration of cytosolic fractions was determined at 280 nm with UV-Visible spectrometer (Nano Drop ND1000 Thermo Fisher Scientific, Waltham, MA).

**Sulfotransferase activity**

Sulfotransferase enzymes present in the cytosolic fraction of liver tissue were incubated with radiolabeled sulfonyl donor \([35S]-3'-phosphoadenosine-5'-phosphosulfate (35S-PAPS)\) and a prototype substrate in 20 mM potassium phosphate (pH 7.0). For these assays PAPS was used at a concentration of 3-6 \(\mu\)M, which is near its Km value. The reaction mixture was incubated for a defined time at 37ºC, stopped by heating in boiling water for 30 sec, and centrifuged at 14,000 \(x\) g for 1 min to pellet the protein. For separation of sulfated products of \(p\)-nitrophenol, estradiol, and dehydroepiandrosterone, the resulting supernate was injected onto Phenomenex Synergi Polar-RP column (50 x 2.00 mm, 4 micron). An appropriate gradient of 20 mM potassium phosphate (pH 2.7) and acetonitrile was used as mobile phase to separate excess 35S-PAPS from 35S-product. For dopamine-sulfate separation, Hypersil Duet C18/SAX column (150 x 4.6 mm, 5 micron, ThermoScientific) with a mobile phase of ammonium bicarbonate (pH 8.0) and acetonitrile was used. Radiolabel was detected and quantified on a flow scintillation analyzer (Packard Bioscience, 500 TR series) with Perkin-Elmer Ultima Flo-M scintillation cocktail. 35S-PAPS was eluted with the solvent front, \(p\)-nitrophenol-sulfate at 1.5 min, dehydroepiandrosterone-sulfate at 5 min, estradiol-sulfate at 4.5 min, and dopamine-sulfate at 3.5 min (Supplemental Figure 1). The dopamine-sulfate produced eluted as a single peak and was not further analyzed for regioisomeric composition.
Immunoblot Analysis

Protein expression of four major hepatic sulfotransferases was quantified by immunoblotting. In order to reduce experimental variability, 38 samples were loaded on a single gel (20 cm x 20 cm) so that all samples were contained in two large gels. This 2-gel set was subsequently transblotted and antibody incubations were conducted such that all 76 samples were treated simultaneously. The 2-gel set was conducted a total of four times for incubation with each of the four anti-SULT isoform antibodies. Cytosolic protein (40 μg protein/well) was resolved by SDS-polyacrylamide gel electrophoresis (12% resolving and 8% stacking gel). Proteins were transblotted onto PVDF membrane (Millipore Corporation, Billerica, MA) at 100 V for 40 min. After washing the membrane with phosphate-buffered saline for 2 hours, it was incubated with primary antibody (anti-SULT1A1, anti-SULT1E1, or anti-SULT1A3) diluted in phosphate-buffered saline with Tween 20 overnight at 4°C. For SULT2A1 detection, the membrane was incubated only for 1 hour with anti-SULT2A1 antibody. Then each membrane was washed and incubated with infrared dye-labeled secondary antibody (Li-Cor, NE) for 1 hour at room temperature in the dark. Infrared signal of Western blot bands were detected and quantified using an Odyssey® Infrared Imaging System (Li-Cor, NE). Antibody selectivity was tested as shown in Supplemental Figure 2.

RNA Extraction

Total RNA from human livers (280-320 mg of each sample) were isolated by phenol-chloroform extraction using RNA Bee reagent (Tel-Test Inc., Friendswood, TX)
according to the manufacturer’s protocol. Total RNA quantification was made by measuring the absorbance at 260 nm in a UV-visible spectrophotometer (NanoDrop ND 1000; Thermo Fisher Scientific, Waltham, MA). RNA integrity was verified by formaldehyde-agarose gel electrophoresis.

**Branched DNA Signal Amplification Assay for mRNA Quantification**

Assay was similar to previously published (Hardwick et al., 2012). All reagents for analysis including oligonucleotide primers designed specifically for each sulfotransferase, lysis buffer, amplifier/label probe diluent, and substrate solution were supplied in the QuantiGene HV signal amplification kit (Panomics, Fremont, CA). Primers designed specifically for each sulfotransferase were diluted in lysis buffer. On the first day, RNA samples diluted to 1 μg/μL were added to each of the 96-well plates containing 50 μL of capture hybridization buffer and 100 μL of diluted probe set. The RNA was allowed to hybridize overnight with a probe set at 53°C. On the second day of the assay, subsequent hybridization steps were followed as mentioned in the manufacturer’s protocol, and luminescence was measured with a Quantiplex 320 branched DNA luminometer interfaced with Quantiplex Data Management Software (version 5.02; Bayer Corp., Diagnostics Div., Tarrytown, NY). The luminescence for each well was reported as relative light units per 10 μg of total RNA.

**Quantitative real-time PCR for mRNA quantification of human SULT1A3**

qPCR was utilized to measure mRNA expression of SULT1A3 which was not available in the QuantiGene Plex used. Total RNA was converted to cDNA, then mRNA levels
were quantified by quantitative real-time PCR using a Roche LightCycler 480 System (Roche Applied Science, Mannheim, Germany). SYBR green chemistry was used, and relative target gene expression was normalized to GAPDH. The SULT1A3 primers used were published by Dooley et al (2000).

Statistical analysis

Raw data from mRNA quantification were normalized to housekeeping gene 60S ribosomal protein L13a (RPL13A). Sulfotransferase activity and normalized mRNA data were log transformed and then plotted as quantile-quantile plots (QQplot) and box plots. The linearity of the points from QQplot suggests that the data were normally distributed. The differences in mRNA quantification and sulfotransferase activity quantification between groups and genders were determined by MANOVA (multivariate analysis of variance) using a significance level of 0.05. Data from SULT protein quantification were normalized to anti-GAPDH quantification and was analyzed by SPSS 19.0 software using ANOVA and considering the difference of $p \leq 0.05$ as statistically significant. The diabetes group, containing only four individuals, was too small for group statistical comparisons and the data were utilized for qualitative comparison only. The remaining groups had sufficient sample size and the log transformation of raw data demonstrated a normal distribution.
Results

I. Liver Disease Affects Sulfotransferase Activity

SULT1A1 (using 4 µM p-nitrophenol), SULT2A1 (10 µM dehydroepiandrosterone), SULT1E1 (20 nM estradiol), and SULT1A3 (10 µM dopamine) activities were determined at concentrations that were selective in human liver cytosol for each isoform (Tabrett and Coughtrie, 2003; Huang et al., 2010; Falany et al., 1995; Yasuda et al., 2007). Incubation time and amount of cytosolic protein to achieve appropriate linear kinetic conditions for each assay were empirically determined. Activity assays for each isoform in each tissue sample were performed in duplicate except for SULT1A3 which was single measurement per tissue sample. Sulfonation was quantified in liver samples from humans diagnosed with steatosis (n=13 individuals), diabetes (n=4), diabetic cirrhosis (n=22), alcohol cirrhosis (n=22) and non-fatty liver (n=20) (Figures 1A, 2A, 3A, 4A). The diabetes group, containing only four individuals, was too small for group statistical comparisons and the data are shown for qualitative comparison only.

Mean SULT1A1 activity was found to be decreased substantially in steatosis, diabetic cirrhosis and alcoholic cirrhosis versus non-fatty control, with no difference of males versus females (Figure 1A and Table 1). For example, steatosis samples contained 42% of non-fatty control activity, diabetic cirrhosis 34% of non-fatty control activity, and alcoholic cirrhosis 18% of non-fatty control activity ($p \leq 0.0001$). It is especially noteworthy that the SULT1A1 activity in alcoholic cirrhosis tissues was so low that it was also significantly decreased ($p \leq 0.02$) from other disease states: a further 42% reduction from steatotic and 52% reduction from diabetic cirrhotic samples. This
indicates that tissues from individuals with cirrhosis caused by alcohol exposure have even lower SULT1A1 activity than tissues from individuals with diabetic cirrhosis (i.e. non-alcoholic).

In contrast to SULT1A1, neither steatotic nor diabetic cirrhotic tissues differed in their SULT2A1 activity versus non-fatty tissues (Figure 2A, Table 1). However, mean SULT2A1 activity was found to be decreased substantially in the alcohol cirrhosis group. For example, alcohol cirrhosis samples contained 47% of non-fatty control activity, 32% of steatosis activity, and 41% of diabetic cirrhosis activity ($p \leq 0.0001$). Gender difference was observed only in diabetic cirrhosis samples with females possessing somewhat higher DHEA-sulfonation than males ($p \leq 0.03$).

SULT1E1 activity in steatotic samples was not significantly reduced versus the non-fatty control samples (Figure 3A, Table 1). However, similar to SULT1A1 and SULT2A1, SULT1E1 activity in the other disease states was significantly reduced versus non-fatty control: 61% diabetic cirrhosis and 27% alcohol cirrhosis. Gender difference was observed in alcohol cirrhosis samples with females possessing somewhat lower estradiol-sulfonation activity than males ($p \leq 0.04$).

Similar to SULT1A1 activity, SULT1A3 activity was found to be decreased substantially in steatosis, diabetic cirrhosis and alcoholic cirrhosis as compared to non-fatty control (Figure 4A, Table 1). For example, steatosis samples contained 65% of non-fatty control activity, diabetic cirrhosis 59% of non-fatty control activity, alcoholic cirrhosis 51% of
non-fatty control activity \((p \leq 0.015)\). It is noteworthy that the SULT1A3 activity in alcoholic cirrhosis tissues was also significantly \((p \leq 0.02)\) decreased from other disease states: 74% of steatotic and 86% of diabetic cirrhotic samples. Gender difference was observed in female alcohol cirrhosis samples, which contained substantially lower SULT1A3 activity than males \((p \leq 0.0001)\). Because the dopamine-sulfate formed was eluted as a single 35S-labeled peak, it was not further analyzed for 3-O- or 4-O-regioisomeric position.

Table 1 summarizes the above described effects of liver disease on human sulfotransferase activity. SULT1A1 and SULT1A3 activities were lower in disease states relative to non-fatty tissues. Alcoholic cirrhotic tissues further contained lower SULT1A1 and 1A3 activities than either of the two other disease states. SULT2A1, on the other hand, was reduced only in alcoholic cirrhotic tissues. SULT1E1 was reduced in both diabetic cirrhosis and alcoholic cirrhosis tissues, relative to non-fatty liver tissues.

Figure 5 illustrates total and relative sulfonation capacity measured in non-fatty and diseased liver tissues. Relative contribution of each isoform to the total sulfotransferase activity points out that SULT1A1 and SULT2A1 carried out more sulfonation than SULT1E1 and SULT1A3 in non-fatty and all disease types. In non-fatty livers, SULT1A1 and SULT2A1 were responsible for 74% and 20% of total sulfonation, whereas SULT1E1 and SULT1A3 together were responsible for only 6% of total sulfonation in non-fatty liver. This is consistent with results published previously (Riches et al., 2009) for non-diseased liver tissue. However, we found that sulfotransferase activity
decreased with increased severity of liver disease from steatotic liver to cirrhosis. When compared to sulfotransferase activity in non-fatty livers, total sulfonation in steatosis samples was only 65%, sulfonation in diabetic cirrhosis samples was 51%, and sulfonation in alcohol cirrhosis was only 25% of total sulfonation in non-fatty control group.

II. Effects of Liver Disease on Sulfotransferase Protein Expression

In addition to sulfotransferase activity, sulfotransferase protein expression was determined by Western blot. We have utilized commercially available anti-SULT1A1, anti-SULT2A1, anti-SULT1E1, and anti-SULT1A3 antibodies in order to detect the protein expression in the same human liver cytosols that we used to measure the sulfotransferase activity. Similar to activity, there was considerable individual variation in protein expression within the disease groups. The expression of four SULT proteins, SULT1A1, SULT2A1, SULT1E1 and SULT1A3, was detected at various quantities in human liver tissues including steatosis (n=14 individuals), diabetes (n=4), diabetic cirrhosis (n=21), alcohol cirrhosis (n=17) and non-fatty livers (n=20) (Figures 1B and 1D, 2B and 2D, 3B and 3D, 4B and 4D). The discrepancy in number of tissues in each group versus activity data were due to limited sample volumes. In contrast to activity and mRNA measurements, many of the immunogenic protein values were below the level of detection. This is not unexpected, because the sensitivities of the antibodies are limited.
In accordance with enzyme activity data, protein expression of SULT1A1 was found to be expressed at substantially higher amounts in non-fatty livers versus diseased livers and the expression was significantly reduced in diseased livers including steatosis, diabetes cirrhosis and alcohol cirrhosis (p ≤ 0.0001) (Figure 1B). SULT2A1 protein expression was not different between groups, although the range of highest to lowest protein was larger in the diseased versus non-fatty tissues (Figure 2B). SULT1E1 and SULT1A3 protein expressions were higher in steatosis samples compared to non-fatty, diabetes cirrhosis and alcohol cirrhosis livers (p ≤ 0.05) (Figure 3B and 4B).

III. Effects of Liver Disease on Sulfotransferase mRNA Expression

Along with sulfotransferase activity and protein expression, we have quantified mRNA expression of SULT1A1, SULT2A1, SULT1E1, SULT1A3, and PAPSS2 (PAPS synthase 2) in the same human liver tissues (Figures 1C, 2C, 3C, and 6). Similar to activity, we observed substantial interindividual variation within disease groups. Specific comparisons are as follows.

SULT1A1 mRNA expression was detected at unexpectedly low levels in all liver samples including non-fatty and disease groups, and there was no statistically significant difference in SULT1A1 mRNA detected between any groups (Figure 1C). SULT2A1 mRNA expression decreased substantially in alcohol cirrhosis group versus non-fatty and versus other disease groups (p ≤ 0.05) (Figure 2C). Gender difference was observed in SULT2A1 mRNA expression, which was found to be less abundant in female than male subjects (p ≤ 0.04). SULT1E1 mRNA expression in the steatosis
group was similar to the non-fatty control group. SULT1E1 expression decreased significantly in diabetes cirrhosis, but not in the alcohol cirrhosis group compared to non-fatty and steatosis groups (p ≤ 0.05) (Figure 3C). SULT1A3 mRNA expression did not show any significant difference between groups. PAPSs2 mRNA expression in steatosis group trended lower than non-fatty control group, but the difference was not statistically significant (Figure 6). However, PAPSs2 expression in diabetic and alcoholic cirrhosis groups was significantly reduced versus non-fatty control and versus steatosis groups (p ≤ 0.0002). A gender difference was observed only in alcohol cirrhosis group, with females having more PAPSs2 mRNA expression than males (p ≤ 0.0001) (Figure 6).
Discussion

Phase-II biotransformation enzymes have an essential role in termination of pharmacologic activity, detoxification, and elimination of xenobiotics from the body by conjugating small molecules to xenobiotics and thus altering their pharmacokinetic characteristics. In order to understand the contribution of these enzymes to the xenobiotic and endobiotic metabolism, it is necessary to elucidate enzymes’ characteristics including their tissue expression, activity, and regulation. Human sulfotransferases have been well-characterized in healthy liver tissues (Riches et al., 2009), but little has been reported in diseased liver tissue (Elekima et al., 2000, Hardwick et al, 2012, Yeo et al., 2010). Thus, in this paper we report sulfotransferase activity and expression in healthy liver tissue versus tissue of individuals diagnosed with steatosis, diabetes, diabetic cirrhosis, and alcohol cirrhosis.

One challenge in profiling these sulfotransferases is whether to focus on functional (activity) measurements or expression measurements at the translational (protein) or transcriptional (mRNA) level. This challenge is especially relevant in the sulfotransferase field because several studies have found weak correlations of activity to immunogenic protein to message (for example, Duanmu et al., 2006, Riches et al., 2009, Hardwick et al., 2012). In our hands, we are most confident in the sulfotransferase activity measures and have emphasized these results throughout the discussion and conclusion sections.
Our results show that, with expected interindividual differences, hepatic expression and activity of sulfotransferases varied between diseased and non-fatty livers. As required for its role in drug metabolism and disposition in liver, SULT1A1 catalyzed the majority of hepatic sulfonation. However, we found that the capacity of SULT1A1 diminished significantly in intact livers with steatosis and in more seriously diseased liver tissue. Furthermore, SULT1A1 protein expression was previously reported to be lost in the liver tissues of patients diagnosed with hepatocellular carcinogenesis (Yeo et al., 2010). This reduction in SULT1A1 sulfonation may result in accumulation of xenobiotics eliminated through sulfate conjugation with subsequent adverse effects and may require reevaluation of safe dosages/exposures for individuals with fatty liver disease.

SULT2A1 activity (DHEA sulfonation) was found to be decreased significantly only in alcohol cirrhosis group, but not in steatotic or diabetic cirrhotic livers as compared to non-fatty controls. Steatotic livers resemble early stage of NAFLD, where fat accumulation occurs without inflammation, fibrosis or hepatocyte changes (Hashimoto and Tokushige, 2011). Interestingly, DHEA sulfonation of diabetic cirrhosis samples resulted in two subgroups— one resembling the high sulfonation in non-fatty livers and the other resembling the diminished sulfonation in livers with alcohol cirrhosis. A plausible explanation is that the diabetic cirrhosis group has a wide range of disease state including livers with the onset of inflammation and others with severe inflammation, where the liver cells have been replaced by the scar tissue. This observation will need further study.
Circulating levels of DHEA-sulfate in serum have been reported in patients diagnosed with NAFLD in the absence of inflammation and with advanced NAFLD. Plasma DHEA-sulfate levels were found to be increased with elevated ALT levels in NAFLD patients (Koga et al., 2011; Völzke et al., 2010). This may indicate a compensatory increase of adrenal secretion and sulfonation of DHEA in order to protect liver. Indeed, DHEA was able to inhibit inflammatory cytokines, hepatocyte apoptosis, and decrease serum ALT levels in a mouse model of hepatitis (Yoneda et al., 2004). However, with advanced NAFLD, where NASH is present, DHEA-sulfate levels have been detected to be decreased (Charlton, 2008). This might be due to decreased hepatic sulfonation or reduced efflux of DHEA as a result of impaired activity of enzymes and transporters, respectively.

Administration to rodents of DHEA at physiological concentrations and DHEA-sulfate at much lower concentrations increased the size and number of peroxisomes present in liver. (Peters et al., 1996). To determine whether PPAR regulated the peroxisomal gene induction by DHEA-sulfate, mice lacking functional PPAR alpha gene were administered DHEA-sulfate and clofibrate, a known PPAR agonist. Both treatments induced peroxisome proliferator response in wild type mice, but not in PPAR alpha-deficient mice (Peters et al., 1996). In addition, clofibrate treatment of primary cultured human hepatocytes increased human SULT2A1 mRNA, protein and enzymatic activity (Fang et al., 2005). Therefore, PPAR contributes to DHEA-sulfate stimulated hepatic peroxisomal gene induction. Thus, the decreased SULT2A1 expression in alcohol cirrhosis livers
could be associated with PPAR alpha expression which might be a potential target in order to maintain hormonal homeostasis in diseased liver tissue.

In addition to DHEA, SULT2A1 catalyzes metabolism of testosterone; whereas SULT1E1 catalyzes elimination of estrogens (Luu-The et al., 1996). Thus, both sulfotransferases can regulate the concentration of biologically active sex steroids in the liver and systemic circulation. Our results indicate decreased activity of SULT1E1 in livers with cirrhosis (both alcoholic and diabetic) versus non-fatty and steatotic livers. This may contribute to elevated levels of estrogens (estradiol and estrone) and continuous activation of estrogen receptor in cirrhotic livers. Elevated levels of systemic estrogen have been reported in patients with liver cirrhosis (Montalto et al., 1997; Nakamuta et al., 1994; Becker et al., 1991). In addition, gynecomastia has been commonly observed in male patients with cirrhosis due to impaired estrogen metabolism (Narasaka, 2000). However the biological role of liver SULT1E1 is unclear at this time.

In contrast to the excess estrogen associated with cirrhosis, low estrogen conditions may play a role in development of NAFLD. NAFLD has been found to be more prevalent in men, postmenopausal women and women with polycystic ovary syndrome than in premenopausal women suggesting the hepatoprotective roles of estrogens in healthy liver (Gutierrez-Grobe et al., 2010; Shimizu and Ito, 2007). Hepatic steatosis became evident in the aromatase deficient mouse, which cannot produce estrogen, and steatosis was attenuated after estradiol treatment (Nemoto, et al., 2000). In contrast,
tamoxifen-treated non-obese, non-diabetic patients were reported to develop progressive steatosis, which occasionally induced NASH and liver cirrhosis (Akhondi-Meybodi et al., 2011).

Previous studies could not detect immunogenic SULT1A3 protein in human liver cytosols (Riches et al., 2009; Teubner et al., 2007); however, we were able to detect protein expression of SULT1A3 in non-fatty and diseased human livers. The lack of previous detection of hepatic SULT1A3 protein expression could be related to binding affinity of the antibodies used in previous studies. Bands were detected corresponding to the predicted molecular weight for SULT1A3 protein with a newly available commercial antibody that was highly specific to SULT1A3 protein and did not recognize recombinant expressed SULT1A1 (Supplemental Table 2, Supplemental Figure 2). In addition to expression, we were able to detect dopamine sulfonation in non-fatty and diseased livers. Our results indicate decreased activity of SULT1A3 in steatotic and cirrhotic (both diabetic and alcoholic) liver tissues compared to non-fatty livers. This may result in reduced sulfonation of catecholamines in liver disease; however, the understanding of the role of phenylethylamine or catecholamine action in hepatobiliary-intestinal function is minimal.

The universal sulfate donor PAPS (3’-phosphoadenosine-5’-phosphosulfate) is synthesized by PAPS synthase (PAPSs), a bifunctional protein including ATP-sulfurylase and APS-kinase activities (Venkatachalam, 2003). mRNA expression of PAPSs in diabetic and alcoholic cirrhosis samples was significantly reduced versus non-
fatty control and versus steatosis samples indicating that cofactor deficiency may be an additional factor contributing to low sulfonation capacity in individuals with liver cirrhosis.

The diminished sulfotransferase expression and activity in liver disease could be related to inflammation (Bryan et al., 2013). Previous studies have shown the induction of proinflammatory cytokines with diet-induced obesity, which resulted in reduced levels of drug metabolism enzymes including sulfotransferases (Ghose et al., 2011). Rodent models revealed LPS (lipopolysaccharide)-induced inflammation caused suppression of Sult1a1 and Sult2a1 gene expression (Shimada et al., 1999; Kim et al., 2004). Accordingly, severe inflammation in alcohol induced cirrhosis may contribute to the dramatically low levels of sulfotransferases found in cirrhotic human livers.

Use of human donor tissue often carries the concern of tissue integrity. Because our data showed decreased SULT activity in diseased liver tissue, we chose to measure an additional parameter which is known to be upregulated by liver disease (NAD(P)H:quinone oxidoreductase 1, NQO1) (Hardwick et al., 2010). NQO1 is a cytosolic protein that catalyzes the two-electron reduction of reactive quinones, which are capable of producing reactive oxygen species (Jaiswal, 2000). Our results showed an increasing trend in NQO1 activity with the progression of NAFLD (Supplemental Figure 3), in agreement with Hardwick et al. (2010), indicating the potential to measure increased enzyme activity in the samples analyzed.

In summary, in this study we characterized the alteration of sulfotransferases at transcriptional and translational levels in human livers diagnosed with steatosis,
diabetes, and cirrhosis. Both alcohol- and diabetes-induced cirrhosis showed profound reduction of major hepatic sulfotransferase isoforms (SULT1A1, SULT1E1, and SULT1A3), while the milder steatosis reduced only SULT1A1 and SULT1A3. The current study cannot confirm causation versus association, but may lead to important consequences such as xenobiotic accumulation and toxicity due to diminished SULT1A1, alteration in androgen synthesis and metabolism due to modified SULT2A1, alteration in estrogen metabolism due to modified SULT1E1, or accumulation of catecholamines due to diminished SULT1A3 levels in diseased liver.
**Authorship Contributions**

Conducted research design: Yalcin, King.

Conducted experiments: Yalcin, More, and Neira.

Contributed new reagents or analytical tools: Slitt

Performed data analysis: Yalcin and Lu.

Wrote or contributed to the writing of the manuscript: Yalcin, King, Slitt, Cherrington.
References


Footnotes

This study was supported in part by the University of Rhode Island (URI) Foundation (RSK), the URI Council for Research (RSK), a URI Graduate Fellowship (EBY) and a Summer Undergraduate Research Fellowship from RI-INBRE (KLN). This work also was supported in part by the National Institutes of Health [5R-01ES-016042-03], [3R-01ES-016042-2S2], and [5K-22ES-013782-03] (ALS), and by the Rhode Island IDeA Network of Biomedical Research Excellence [P20RR016457-10] from the National Center for Research Resources, National Institutes of Health (RSK, ALS).

A portion of this work was presented at the International Society for the Study of Xenobiotics (ISSX) meeting, 2011, October 16-20, Atlanta, Georgia, GA.

Address correspondence to: Roberta S. King, Ph.D., Department of Biomedical and Pharmaceutical Sciences, College of Pharmacy, University of Rhode Island, 7 Greenhouse Road, Kingston, RI 02881

Email: rking@uri.edu
Figure Legends

Figure 1. Expression and activity of SULT1A1 in non-fatty (control) and diseased human livers. Each data point represents a single tissue (average of determinations) categorized by gender and disease type (n=20 for nonfatty, n=13-14 for steatosis, n=4 for diabetes, n=21-22 diabetic cirrhosis, and n=17-22 for alcohol cirrhosis). Females are displayed in red, males in blue color. Statistically significant differences (p < 0.05) between non-fatty and diseased livers are represented by asterisk (*). Statistically significant differences (p < 0.05) between alcohol cirrhosis and other groups are represented by “#”. (A) Enzyme activity was determined by incubating 4 μM p-nitrophenol with human liver cytosols for 30 min in the presence of the 35S-labeled cofactor PAPS. (B) Protein expression of SULT1A1 was quantified by Western blotting analysis in human livers. (C) Messenger RNA expression of SULT1A1 was quantified by branched DNA signal amplification assay (Affymetrix Inc., CA, USA). (D) Membranes loaded with 40 μg protein and probed with anti-SULT1A1 antibody. Lane numbers refer to sample ID as noted in Supplemental Table 1.

Figure 2. Expression and activity of SULT2A1 in non-fatty (control) and diseased human livers. Each data point represents a single tissue (average of determinations) categorized by gender and disease type (n=20 for nonfatty, n=13-14 for steatosis, n=4 for diabetes, n=21-22 diabetic cirrhosis, and n=17-22 for alcohol cirrhosis). Females are displayed in red, males in blue color. Statistically significant differences (p < 0.05) between alcohol cirrhosis and other groups are represented by “#”. (A) Enzyme activity was determined by incubating 10 μM dehydroepiandrosterone with human liver cytosols.
for 30 min in the presence of the 35S-labeled cofactor PAPS. (B) Protein expression of SULT2A1 was quantified by Western blotting analysis in human livers. (C) Messenger RNA expression of SULT2A1 was quantified by branched DNA signal amplification assay (Affymetrix Inc., CA, USA). (D) Membranes loaded with 40 μg protein and probed with anti-SULT2A1 antibody. Lane numbers refer to sample ID as noted in Supplemental Table 1.

Figure 3. Expression and activity of SULT1E1 in non-fatty (control) and diseased human livers. Each data point represents a single tissue (average of determinations) categorized by gender and disease type (n=20 for nonfatty, n=13-14 for steatosis, n=4 for diabetes, n=21-22 diabetic cirrhosis, and n=17-22 for alcohol cirrhosis). Females are displayed in red, males in blue color. Statistically significant differences (p < 0.05) between alcohol cirrhosis and other groups are represented by “#”. Statistically significant differences (p < 0.05) between steatosis and other groups are represented by “+”. (A) Enzyme activity was determined by incubating 20 nM 3H-labeled estradiol with human liver cytosols for 30 min in the presence of the 35S-labeled cofactor PAPS. (B) Protein expression of SULT1E1 was quantified by Western blotting analysis in human livers. (C) Messenger RNA expression of SULT1E1 was quantified by branched DNA signal amplification assay (Affymetrix Inc., CA, USA). (D) Membranes loaded with 40 μg protein and probed with anti-SULT1E1 antibody. Lane numbers refer to sample ID as noted in Supplemental Table 1.
Figure 4. Expression and activity of SULT1A3 in non-fatty (control) and diseased human livers. Each data point represents a single tissue (average of determinations) categorized by gender and disease type (n=20 for nonfatty, n=13-14 for steatosis, n=4 for diabetes, n=21-22 diabetic cirrhosis, and n=17-22 for alcohol cirrhosis). Females are displayed in red, males in blue color. Statistically significant differences (p < 0.05) between non-fatty and diseased livers are represented by asterisk (*). Statistically significant differences (p < 0.05) between steatosis and other groups are represented by “+”. (A) Enzyme activity was determined by incubating 10 μM dopamine with human liver cytosols for 30 min in the presence of the 35S-labeled cofactor PAPS. (B) Protein expression of SULT1A3 was quantified by Western blotting analysis in human livers. (C) Messenger RNA expression of SULT1A3 was quantified by quantitative real-time PCR using a Roche LightCycler 480 System (Roche Applied Science, Mannheim, Germany). (D) Membranes loaded with 40 μg protein and probed with anti-SULT1A3 antibody. Lane numbers refer to sample ID as noted in Supplemental Table 1.

Figure 5. Total and relative sulfonation of SULT1A1, SULT2A1, SULT1E1, and SULT1A3 in non-fatty (control) and diseased human livers. Total sulfotransferase activity is indicated by colored bars in each tissue type. The percentage of sulfotransferase activities of four major isoforms were calculated for non-fatty (control) group.

Figure 6. PAPSs2 mRNA expression in humans in non-fatty (control) and diseased human livers. PAPSs2 mRNA expression was quantified by branched DNA
signal amplification assay (Affymetrix Inc., CA, USA). Asterisks (*) represent statistically significant differences ($p < 0.05$) between non-fatty and diseased livers.
Table 1. Summary of changes in sulfonation activity of the four major sulfotransferases in diseased human livers versus non-fatty tissue.

<table>
<thead>
<tr>
<th>Disease state</th>
<th>SULT1A1</th>
<th>SULT1A3</th>
<th>SULT1E1</th>
<th>SULT2A1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steatosis</td>
<td>↓↓</td>
<td>↓</td>
<td>↔</td>
<td>↔</td>
</tr>
<tr>
<td>Diabetes cirrhosis</td>
<td>↓↓↓</td>
<td>↓</td>
<td>↓</td>
<td>↔</td>
</tr>
<tr>
<td>Alcohol cirrhosis</td>
<td>↓↓↓↓</td>
<td>↓↓</td>
<td>↓↓↓</td>
<td>↓↓</td>
</tr>
</tbody>
</table>

↔ indicates no change from the non-fatty, non-diseased liver
↓ indicates 59-65% of activity in non-fatty, non-diseased liver
↓↓ indicates 42-51% of activity in non-fatty, non-diseased liver
↓↓↓ indicates 27-34% of activity in non-fatty, non-diseased liver
↓↓↓↓ indicates 18% of activity in non-fatty, non-diseased liver
Figure 1

A. SULT1A1 activity (pNP sulfonation)

B. SULT1A1 protein

C. SULT1A1 mRNA

* difference between non-fatty and diseased livers (p < 0.05)
# difference between alcohol cirrhosis and other groups (p < 0.05)
Figure 2

A) SULT2A1 activity (DHEA sulfonation)

B) SULT2A1 protein

C) SULT2A1 mRNA

# difference between alcohol cirrhosis and other groups (p < 0.05)

D) Western Blot
Figure 6

[Graph showing average RLU/10 ug of total RNA across different conditions: Nonfatty, Steatosis, Diabetes, Diabetes Cirrhosis, Alcohol Cirrhosis. Each condition has data points with error bars indicating variability. Asterisks indicate statistically significant differences.]