Oxidative Modification of Rat Sulphotransferase 1A1 Activity in Hepatic Tissue Slices
Correlates with Effects on the Purified Enzyme

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Oxidative modification of rSULT1A1 in hepatic tissue slices

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Abbreviations: GSH: reduced glutathione; GSSG, glutathione disulfide, PAPS, adenosine 3'-phosphate 5'-phosphosulfate; DTT, dithiothreitol; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); SULT, sulfotransferase; 7-HC, 7-hydroxycoumarin; 7-HCS, 7-hydroxycoumarin sulfate; LDH, lactate dehydrogenase; TBHP, tert-butyl hydroperoxide
Abstract

Mammalian cytosolic sulfotransferases (SULTs) catalyze the sulfation of xenobiotics as well as numerous endogenous molecules. The major aryl (phenol) sulfotransferase in rat liver, rSULT1A1, has been extensively utilized as a model enzyme for understanding the catalytic function of SULTs. Previous studies have shown that purified rSULT1A1 displays significant catalytic changes in the presence of glutathione disulfide (GSSG) and other oxidants. In the present study, the effects of diamide (1,1'-azobis(N,N-dimethylformamide)) and tert-butyl hydroperoxide (TBHP) on the activity of rSULT1A1 within rat hepatic slices were compared with the effects of these oxidants on a homogeneous preparation of the enzyme. Precision-cut hepatic slices were incubated with 10 µM 7-hydroxycoumarin (7-HC) in the presence of varied concentrations of either diamide or TBHP. Analysis of the 7-hydroxycoumarin sulfate released into the incubation medium indicated that both oxidants significantly increased the sulfation of 7-HC, and this occurred at optimum concentrations of 5 µM and 10 µM, respectively. Cellular GSH and GSSG levels in the hepatic slices were not significantly altered from control values at these concentrations of diamide and TBHP. Exposure of homogeneous rSULT1A1 to diamide or TBHP also increased the rate of sulfation of 7-HC, although the optimum concentrations of diamide and TBHP were lower (50-fold and 100-fold, respectively) than those required for effects with the hepatic slices. Thus, these results indicate that both diamide and TBHP may modify the rSULT1A1 within intact cells in a manner similar to that observed with the homogeneous purified enzyme.
Introduction

The mammalian cytosolic sulfotransferases (SULTs) contribute to the metabolism of various drugs, carcinogens, environmental contaminants, and other xenobiotics as well as functioning in the metabolism of endogenous steroids, bile acids, catecholamines, and iodothyronines (Jakoby and Ziegler, 1990; Glatt, 2000; Chapman et al., 2004; Gamage et al., 2006; Alnouti, 2009; Duffel, 2010). The SULTs comprise a superfamily of enzymes that have been organized into families and subfamilies (Blanchard, 2004 #1498) based on sequence similarity. While there are distinct specificities for substrates and inhibitors among SULT families, there is a high degree of structural homology among these enzymes (Negishi et al., 2001), as well as significant overlap in specificity for some substrates (Gamage et al., 2006; Duffel, 2010).

Among the SULTs that have been extensively used in studies on mechanism, specificity, and inhibition, rSULT1A1, the major family 1 sulfotransferase in rat liver, has been particularly useful in studies that focus on details of the chemical and kinetic mechanisms of reactions catalyzed by sulfotransferases (Duffel and Jakoby, 1981; Duffel et al., 2001; Chapman et al., 2003). One of the more intriguing aspects of the mechanism of rSULT1A1 is the sensitivity of the enzyme to changes in its redox environment (Marshall et al., 2000). Studies on homogeneous preparations of the enzyme have indicated that treatment with thiol-oxidants such as diamide and glutathione disulfide result in increases in the specific activity of rSULT1A1 with 4-nitrophenol as substrate (Marshall et al., 1997; Marshall et al., 1998; Marshall et al., 2000). In studies with glutathione disulfide as the oxidant these effects were clearly shown to be due to sequential oxidation of cysteine residues in rSULT1A1, with an initial formation of a glutathione-protein mixed disulfide involving cysteine-66,

It has been proposed that this sensitivity of the rSULT1A1 to its redox environment could have significant implications for sulfation of xenobiotics as well as physiological substrates under conditions of oxidative stress, wherein Cys66 could serve as a redox switch regulating the specificity and kinetics of the enzyme (Marshall et al., 1997; Marshall et al., 2000; Duffel et al., 2001; Liu et al., 2009). A critical component of evaluating this possibility is to determine whether oxidation of the enzyme can regulate the rate of reaction within an intact cellular environment. Thus, we have tested the hypothesis that diamide and tert-butyl hydroperoxide (TBHP), two oxidants with differing mechanisms for oxidation of protein thiols, exert similar effects on the sulfation of 7-hydroxycoumarin (7-HC) in viable precision-cut hepatic tissue slices from rats as those seen in the sulfation of 7-HC catalyzed by purified recombinant rSULT1A1.
Materials and Methods

Chemicals. Diamide (1,1'-azobis(N,N-dimethylformamide)), tert-butyl hydroperoxide, 7-hydroxycoumarin, 7-hydroxycoumarin sulfate, lactate dehydrogenase (LDH) assay kit, sulfatase (Type H-2 from *Helix pomatia*), adenosine 3',5'-diphosphate (PAP)-agarose, d-saccharic acid 1,4-lactone, and HEPES (N-[2-hydroxyethyl]piperazine-N'-2-ethanesulfonic acid) were purchased from Sigma-Aldrich (St. Louis, MO). Williams' medium E (GIBCO 12551) was obtained from Invitrogen (Carlsbad, CA). Adenosine 3'-phosphate, 5'-phosphosulfate (PAPS) was obtained from Sigma-Aldrich and further purified using a previously described procedure (Sekura, 1981) to greater than 98% purity as judged by HPLC. All other chemicals used were of the highest chemical purity commercially available.

Preparation of Liver Slices. Male Sprague Dawley rats (280-295 g) were obtained from Harlan Laboratories (Indianapolis, IN). The animals had free access to food and water, and were allowed to acclimate for one week after arrival at the University of Iowa. All protocols were in compliance with the University of Iowa Animal Care and Use Committee and relevant guidelines of the National Research Council for the Care and Use of Laboratory Animals in Research. Following carbon dioxide euthanasia of the rat, the liver was removed and immediately placed in a modified Krebs-Henseleit buffer (Barr et al., 1991) at 4°C. This buffer (buffer A) contained 6.87 g NaCl, 0.4 g KCl, 0.11 g MgCl₂, 0.10 g NaH₂PO₄·H₂O, 2.1 g NaHCO₃, 2.0 g glucose, 0.37 g CaCl₂·2H₂O, and 5.96 g HEPES per liter (adjusted to pH 7.5 with 0.1 N NaOH). Livers were dissected into lobes, and 8.0 mm internal diameter cores were produced using a motor-driven cylindrical stainless steel corer (Alabama...
Research and Development, Munford, AL). The tissue cores were continuously maintained in ice-cold buffer A until the liver slices were prepared within 2h. Cores were placed into a Krumdieck tissue slicer (Alabama Research and Development, Munford, AL), and tissue slices of approximately 250µm thickness were generated by movement of a weighted tissue core over an oscillating blade. The resulting slices were carried to a collection chamber by a stream of ice-cold buffer A.

Cryopreservation of Liver Slices. After preparation, slices were washed two times with a cryopreservation medium consisting of Williams’ Medium E supplemented with 12% (v/v) DMSO. Tissue slices (two per tube) were placed in 2-ml microcentrifuge tubes (Seal-Rite natural polypropylene; USA Scientific, Ocala, FL) containing 1.0 ml of cryopreservation medium and kept in -20°C for 6 h, after which the tubes were transferred to storage at -70°C. The slices were thawed by placing them at -20°C for 2 h, followed by transferring the tubes to a water bath at 37°C and shaking for 2 minutes. Slices were then removed from the cryopreservation medium and washed three times with buffer A. The viability of the tissue slices was assessed by LDH release, and this was examined for slices both with and without incubation with 7-HC and oxidant as described below.

Assay of 7-Hydroxycoumarin Sulfation

Studies on the sulfation of 7-HC were carried out utilizing a modification of a previously described method (Thohan et al., 2001). Two cryopreserved liver slices were placed inside a 20-ml glass liquid scintillation vial (Research Products International, Mt. Prospect, IL) containing 2.0 ml of buffer A, that was supplemented with 10 µM 7-HC and various indicated concentrations of diamide or TBHP. The
open vials were placed on a rotating (1 rpm) incubation platform (Alabama Research and Development, Munford, AL) that was contained inside a cell culture incubator (NAPCO 8000DH, Thermo Fisher, Marietta, OH) maintained at 37°C in the presence of 5% CO₂. After 2 h incubation, one ml of the incubation buffer was mixed with 9 ml of an ether/isoamyl alcohol mixture (ether/IAA, 1:0.014) and the extraction mixture was then placed on a horizontal reciprocating shaker for 15 min. The organic phase, containing non-conjugated 7-HC, was then separated, the ether removed by evaporation with a stream of nitrogen, and the residue dissolved in 3 ml of glycine/NaOH buffer (0.2 M; pH, 10.3). The 7-HC in this sample was determined by fluorescence spectroscopy (Perkin Elmer LS55 spectrofluorimeter) with excitation and emission wavelengths at 375 nm and 450 nm, respectively. Authentic standards of 7-HC were used to establish a standard curve (0.078–1.25 μM).

The aqueous phase (1.0 ml) from the ether/IAA extraction was mixed with 1.0 ml of 0.4 M sodium acetate buffer, pH 4.8, to provide a solution with a final pH of 6.8. Following addition of sulfatase (20 U/ml final concentration) and D-saccharic acid 1,4-lactone (20 mM final concentration for inhibition of any residual glucuronidase activity), each sample was incubated for 2 h at 37°C to hydrolyze the 7-HCS to 7-HC. Reactions were terminated by addition of 9 ml of ether/IAA and the mixture was placed on a horizontal reciprocating shaker for 15 min. The organic phase, containing the 7-HC that had been formed by sulfatase-catalyzed hydrolysis of 7-HCS, was then removed, the solvent evaporated, and the residue dissolved in 3 ml of glycine/NaOH buffer (0.2 M; pH 10.3). The resulting 7-HC was determined by fluorescence spectroscopy as outlined above.
The formation of 7-HCS was normalized to the total protein content of the liver slices in the incubation. Following each tissue slice incubation, the tissue was homogenized in buffer A, and its protein content, as well as that of a sample of the incubation medium, was determined using the modified Lowry procedure (Bensadoun and Weinstein, 1976) with bovine serum albumin as standard.

**Determination of cellular viability in the liver slices.** The effect of 7-HC, diamide, and TBHP on cellular viability was examined by measurement of lactate dehydrogenase (LDH) released into the medium. This was carried out using a standard kit for LDH analysis (Tox-7, Sigma-Aldrich) according to the manufacturer’s instructions. The change in absorbance at 490 nm was quantified using a SpectraMAX 190 plate-reader (Molecular Devices). LDH activities in both the medium and the tissue homogenate were obtained, and values of LDH in the medium were normalized as a percentage of total content of the LDH in the slices and in the incubation medium found in the absence of 7-HC and oxidant. LDH release was determined both with and without a 2 h incubation of the tissue slices under the above-described reaction conditions.

**Sulfation of 7-Hydroxycoumarin by purified rSULT1A1.** Recombinant rSULT1A1 was expressed in *Escherichia coli* BL21(DE3) cells as described previously (Chen et al., 1992). The extraction of rSULT1A1 from the cells and purification by PAP-agarose affinity chromatography was carried out as recently described (Liu et al., 2009). Homogeneity of the resulting rSULT1A1 was confirmed by SDS-PAGE (10% gel) with Coomassie brilliant blue R-250 staining. The purified enzyme was stored at -70°C in pH 7.5 buffer containing 10 mM Tris-HCl, 0.25 M sucrose, 10% (v/v)
glycerol, 1 mM dithiothreitol, 1 μM pepstatin A, and 3 μM antipain. Dithiothreitol and other small thiols were removed by pressure filtration dialysis at 4°C (Amicon PM10 membrane; Millipore Corporation, Billerica, MA) using the above buffer without dithiothreitol. The removal of dithiothreitol was verified using an assay for soluble thiols that employed DTNB (5,5'-dithiobis(2-nitrobenzoic acid)) (Jocelyn, 1987). The purified rSULT1A1 (2.5 μg) was pre-incubated with various concentrations of diamide or TBHP (0.01, 0.1, 1.0, 10 and 100 μM) in a total volume of 0.5 ml of buffer A for 1 h at 25°C. The concentrations of TBHP utilized were verified with a previously described assay for lipid hydroperoxides (Shertzer et al., 1992; Mihaljevic et al., 1996). A 0.4 ml aliquot of the enzyme (i.e., 2.0 μg) that had been pre-treated with either diamide or TBPH was then incubated at 37°C for 30 min in a total volume of 0.5 ml of buffer A containing 10 μM 7-HC and 200 μM PAPS. Reactions were terminated by adding 6 ml of ethyl ether/isoamyl alcohol (ether/IAA, 1:0.014), and the extraction mixture was then placed on a horizontal reciprocating shaker for 15 min. Determinations of the 7-HC in the organic phase and 7-HCS in the aqueous phase were carried out by the same analytical procedure described above for 7-HC and 7-HCS in the hepatic tissue slice incubations.

**Determination of Intracellular GSH and GSSG.** Following incubation of liver slices with 10 μM 7-HC and varying concentrations of diamide, an aliquot (0.3 ml) of each incubation medium was mixed with 0.3 ml of 5% (w/v) 5-sulfosalicylic acid. Individual hepatic slices were rapidly rinsed with buffer A, and both slices were immediately homogenized with 1.0 ml of 5% (w/v) 5-sulfosalicylic acid. The samples were then centrifuged to remove precipitating proteins, and the supernatant fractions were assayed for total glutathione content as described earlier (Anderson, 1985).
The concentration of glutathione disulfide (GSSG) was determined by adding 20 µl of a 1:1 mixture of 2-vinylpyridine and ethanol to 100 µl of the tissue homogenate, and incubating for 2 h at room temperature prior to determining the concentration of GSSG as described previously (Griffith, 1980). The sulfosalicylic acid-precipitated protein was resuspended in 0.1 N NaOH, and the protein concentration was determined using a BCA Assay Kit (Thermo Scientific, Rockford, IL) with bovine serum albumin as standard. All glutathione determinations were normalized to the protein content of the assay.

**Statistical analysis.** Incubations to determine the rate of 7-HCS formation and the content of GSH and GSSG were conducted in triplicate. For studies on hepatic slices the three determinations are derived from separate animals (two slices from each animal in each incubation mixture). Experiments on viability of the hepatic slices were also carried out in this way. Data are presented as the mean ± S.E of determinations from three rats. Results were compared by one-way ANOVA followed by Bonferroni multiple-comparison analysis. A value of p < 0.05 (denoted with *) was used to define statistical significance.
Results

Effects of Diamide and TBHP on Cellular Viability of Liver Slices. The viability of precision-cut hepatic slices under the conditions of incubation with 7-HC was assessed by determining the release of lactate dehydrogenase (LDH) into the incubation medium. As shown in Figure 1, release of LDH is reported as a percentage of the total LDH determined in the incubation medium and the liver slice. Slices from the livers of three rats were incubated for 2 h at 37°C in the presence of 10 µM 7-HC with and without addition of various concentrations of diamide ranging from 1µM to 80 µM. At concentrations of diamide up to 20 µM there were no significant increases in the release of LDH compared to the control group (Figure 1A). In contrast, diamide at concentrations of 40 µM and 80 µM exhibited decreases cellular viability as seen by an increase in release of LDH from the slices. When the hepatic slices were incubated at 37°C with 10 µM 7-HC for 2 h in the presence or absence of TBHP (concentrations up to 80 µM), no significant change in release of LDH was observed.

Effects of Diamide on the Sulfation of 7-HC catalyzed by Purified rSULT1A1 and by Hepatic Slices. Upon incubation of hepatic slices from three rats, diamide significantly increased the rate of sulfation of 7-HC at a concentration of 5 µM (Figure 2A). As the concentration of diamide was increased, the rate of sulfation catalyzed by the tissue slices returned to control values, with a significant decrease occurring at a concentration of 80 µM. This effect of diamide on sulfation in hepatic slices was compared to the effect of this oxidant on homogeneous rSULT1A1 incubated with 7-HC under identical conditions to those utilized for the tissue slices. These experiments utilized homogeneous recombinant rSULT1A1 that had been subjected
to removal of all small thiols and incubated for 1 h at 25 °C with the indicated concentration of diamide as described in Materials and Methods. Following this pre-treatment of the rSULT1A1 with diamide, the enzyme was diluted into an assay containing 10 µM 7-HC and 200 µM PAPS in the same buffer (Buffer A) utilized for the tissue slice studies. After 30 min incubation at 37 °C, the 7-HCS formed was analyzed by the same method used for the tissue slices. As seen in Figure 2B, there were significant increases in the rate of sulfation of 7-HC catalyzed by the homogeneous rSULT1A1 following incubation with either 0.1 µM or 1.0 µM diamide, with a significant decrease in specific activity at 100 µM diamide.

Effects of TBHP on the Sulfation of 7-HC catalyzed by Purified rSULT1A1 and by Hepatic Slices. As was seen with diamide, treatment of either hepatic slices or purified rSULT1A1 with TBHP caused an increase in the rate of sulfation (Figure 3) that then decreased with increasing concentration of oxidant. When hepatic slices from three rats were treated with increasing concentrations of TBHP, the rate of sulfation of 10 µM 7-HC was significantly increased at concentrations of either 10 µM or 20 µM TBHP in the incubation medium. In experiments using the same methodology as described above for the study of the effect of diamide on purified rSULT1A1, pretreatment of the enzyme with TBHP (instead of diamide), followed by dilution into an assay for sulfation of 10 µM 7-HC, indicated that pretreatment with 0.1 µM or 1.0 µM TBHP resulted in an increase in specific activity of the enzyme. Further increases in the concentration of TBHP (i.e., use of either 10 µM or 100 µM) resulted in rates of sulfation that were not significantly different from the control values.
Effect of Diamide and TBHP on the Content of GSH and GSSG in Hepatic Slices. Since one consequence of treatment of hepatic tissue slices with either diamide or TBHP might be an alteration in the cellular ratio of glutathione to glutathione disulfide (GSH/GSSG), we examined the effect of treatment of these oxidants on the concentrations of GSH and GSSG in the slices. Hepatic slices from three rats were challenged with varying concentrations of either diamide or TBHP, such that both the increase and decrease in sulfation seen with these oxidants were included. As seen in Figure 4A, neither the concentration of GSH or GSSG in the hepatic slices deviated significantly from control values upon treatment with diamide at concentrations of 1.0 µM, 5 µM, and 10 µM. Although there was pronounced inter-individual variability in the tissue concentrations of GSH observed upon treatment with TBHP (Figure 4B), these differences were not statistically significant. Moreover, there was also no statistically significant alteration in the overall tissue concentration of GSSG at these concentrations of TBHP (Figure 4B).
Discussion

The oxidation of key cysteine residues in SULTs provides a potential mechanism for reversible regulation of the specificity and rate of catalysis for these enzymes. While this was first described with purified rSULT1A1 (Marshall et al., 1997), investigations of cytosolic and purified preparations of human estrogen sulfotransferase, hSULT1E1, have indicated that the catalytic function of this SULT can also be altered by treatment with GSSG (Maiti et al., 2007). Additionally, studies with rats continuously exposed to hyperoxic conditions (i.e., >95% oxygen) have shown that there was an increase in the rate of sulfation of 2-naphthol catalyzed by subsequently isolated hepatic and lung cytosolic fractions (Maiti et al., 2005). Although previous experiments with both cytosolic and purified preparations of SULTs indicated that oxidation of key cysteines in these enzymes can regulate catalytic activity, direct comparison of the effects of specific thiol oxidants on a homogeneous SULT with the effects of the same oxidants on the enzyme present in an intact cellular environment had not been previously explored.

The major family 1 sulfotransferase in the livers of male Sprague-Dawley rats, rSULT1A1, represents a useful model SULT for comparison of mechanistic studies on the purified enzyme with effects seen during modification of the enzyme in hepatic tissue. The formation of disulfide bonds in rSULT1A1 affects the specificity and pH optimum of the enzyme for some substrates (Marshall et al., 1997; Marshall et al., 2000). Detailed studies on the mechanism of these effects have shown that the rate-determining step of the reaction, namely the breakdown of a non-productive enzyme-PAP-phenol complex, is regulated by the formation of a glutathione-Cys66...
mixed disulfide and the Cys66-Cys232 disulfide (Marshall et al., 2000; Duffel et al., 2001). Previously published homology modeling of rSULT1A1 (Duffel et al., 2001) showed that either the formation of a Cys66-glutathione adduct or a Cys66-Cys232 intramolecular protein disulfide alters the conformation of the PAPS/PAP binding site, but formation of a Cys232-glutathione adduct does not (Marshall et al., 1997). The formation of these disulfide bonds involving Cys66 destabilizes the inhibitory dead-end ternary complex and increases the rate of reaction. However, further alteration of the structure of the enzyme through cysteine oxidation results in a decrease in reaction velocity (Marshall et al., 1997; Marshall et al., 2000; Duffel et al., 2001). It is also important to note in this context that previous mechanistic studies have shown that the structure of the phenolic substrate is important in the formation of the enzyme-PAP-phenol dead end complex (Marshall et al., 2000). Thus, the extent to which the rate of reaction is accelerated by disulfide bond formation involving Cys66 will also depend upon the structure of the substrate, and this would become an important component in evaluating the effect of oxidative regulation on the sulfation of individual drugs or other xenobiotics.

In order to compare the oxidative modification of purified rSULT1A1 with the enzyme in hepatic tissue slices, we chose two oxidants with different structural characteristics (Figure 5) and different mechanisms of action. Diamide acts by formation of a protein-protein internal disulfide bond following initial formation of a cysteine-substituted hydrazinedicarboxamide intermediate that rapidly reacts with a nearby cysteine (Kosower et al., 1969; Harris, 1979). In the presence of glutathione, there would be the possibility of forming a protein-glutathione mixed disulfide, but disulfide interchange would then likely also yield a intramolecular protein disulfide
bond. On the other hand, alkyl hydroperoxides like TBHP act through formation of protein sulfenic acid intermediates that can either react with glutathione to form a protein-glutathione mixed disulfide, react with another protein cysteine to form a protein-protein disulfide bond, or react with an amine to form a sulfenylamide (Kettenhofen and Wood, 2010; Roos and Messens, 2011). Our results showed that regardless of the mechanism for cysteine oxidation, there was a similar effect on sulfation of 7-HC in both precision-cut hepatic slices and with the homogeneous rSULT1A1. As expected from the increased potential for alternate sites of interaction in tissue slices as opposed to the purified enzyme, there was an increase in concentration of oxidant needed in order to observe the effect in the tissue slices relative to that required with the purified enzyme.

Since earlier studies had observed the oxidation of purified SULTs with GSSG, we investigated the potential that the effects that we observed in tissue slices might be due to oxidation of soluble GSH to GSSG, thereby changing the cellular ratio of GSSG/GSH. Subsequent disulfide exchange reaction(s) might then result in formation of glutathione-protein or protein-protein disulfide bonds. However, the cellular concentrations of GSSG and GSH did not significantly change upon treatment of the hepatic slices with the concentrations of diamide or TBHP used in these experiments. Therefore, it is likely that the direct effects of these oxidants seen with the purified enzyme preparation are occurring within the intact tissues slices as well. It should be noted, however, that these specific experiments on hepatic slices would not strictly rule out a transient localized alteration in GSSG/GSH with subsequent disulfide exchange. Nevertheless, this was not occurring with the purified rSULT1A1, since no glutathione was present in those incubations. The
changes in catalytic function of rSULT1A1 also did not correlate with release of LDH in to the medium at concentrations of diamide up to 20 µM and concentrations of TBHP up to 80 µM. At the highest concentration of diamide used (i.e., 80 µM), however, it is possible that altered cellular viability, as shown by LDH release, may have contributed to the lower rate of sulfation observed in tissue slices.

Modifications of proteins through formation of intramolecular protein-protein disulfides, glutathione-protein mixed disulfides, and other oxidation products of cysteine are receiving increased attention due to their potential roles in cellular responses to alterations in redox status, and several recent reviews highlight many of these studies (Janssen-Heininger et al., 2008; Dalle-Donne et al., 2009; Jones and Go, 2010). The results presented in this paper provide a direct correlation between in vitro mechanistic studies on a purified sulfotransferase and effects seen upon treatment of the enzyme with the same oxidant and substrate within viable tissue slices. As such, this supplies an important link between studies with the purified sulfotransferase and events that occur within intact cells and tissues. These findings also point to the importance of examining varied cellular concentrations of the oxidant in observation of these effects, a point that is of key importance in interpreting studies that might otherwise be carried out using higher concentrations of thiol oxidants.

While it remains to be determined the extent to which oxidative stress due to metabolism of other xenobiotics or to disease processes within cells might alter the catalytic function of SULTs by oxidative modification, our results point to the need for further examination of the role that such events may have in regulation of SULTs.
Such studies would also be critical for determining the effects of these oxidative events on in vivo metabolic pathways for specific drugs and other xenobiotics where sulfation is important in either metabolic activation or detoxication.
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Authorship Contributions:

Participated in research design: Dammanahalli and Duffel
Conducted experiments: Dammanahalli
Performed data analysis: Dammanahalli and Duffel
Wrote or contributed to writing of the manuscript: Dammanahalli and Duffel
References


Footnote

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Figure Legends

Fig 1. Viability of Tissue Slices upon Treatment with Diamide and TBHP.

The release of LDH from the tissue slices into the medium was determined following incubation in buffer A containing 10 µM 7-hydroxycoumarin with various concentrations of diamide (1A) or TBHP (1B) at 37°C for 2h. The LDH activity released into the medium is expressed as a percentage of the total (medium + tissue slice) LDH. Data are the mean ± S.E (n=3), and * denotes significant difference from the control without diamide or TBHP (p< 0.05).

Fig 2. Effects of diamide on the sulfation of 7-hydroxycoumarin in hepatic slices and with purified rSULT1A1. (2A). Hepatic slices were incubated in buffer A containing 10 µM of 7-hydroxycoumarin with the indicated concentrations of diamide at 37°C for 2h and aliquots of the medium were analyzed for 7-hydroxycoumarin sulfate as described in Materials and Methods. Data represent the mean ± S.E of incubations of two tissue slices from each of three separate rats. (2B) Purified rSULT1A1 enzyme was pre-incubated with the indicated concentration of diamide in a total volume of 0.5 ml of buffer A, pH 7.4, for 1h at 25°C. The diamide-pretreated enzyme was then used in an assay mixture containing 10 µM 7-HC and 200 µM PAPS in buffer A. The assay with purified rSULT1A1 was carried out at 37°C for 30 min. Data are the means ± S.E. of three determinations. In both 2A and 2B, * denotes significant difference from control (p< 0.05).
Fig. 3. Effects of TBHP on the sulfation of 7-hydroxycoumarin in hepatic slices and with purified rSULT1A1. (3A). Hepatic slices were incubated in buffer A containing 10 µM of 7-hydroxycoumarin with the indicated concentrations of TBHP at 37°C for 2h and aliquots of the medium were analyzed for 7-hydroxycoumarin sulfate as described in Materials and Methods. Data represent the mean ± S.E of incubations of two tissue slices from each of three separate rats. (3B) Purified rSULT1A1 enzyme was pre-incubated with the indicated concentration of TBHP in a total volume of 0.5 ml of buffer A at pH 7.4 for 1h at 25°C. The TBHP-pretreated enzyme was then used in an assay mixture containing 10 µM 7-HC and 200 µM PAPS in buffer A. The assay with purified rSULT1A1 was carried out at 37°C for 30 min. Data are the means ± S.E. of three determinations. In both 3A and 3B, * denotes significant difference from control (p< 0.05).

Fig 4. Measurement of GSH and GSSG following treatment of hepatic slices with diamide or TBHP. Liver slices were incubated for 2h with 10µM 7-hydroxycoumarin and the indicated concentrations of either diamide (4A) or TBHP (4B). Concentrations of GSH and GSSG in the slices were determined as described in Materials and Methods. Results are the means of ± S.E. of three independent determinations, and * denotes significant difference (p< 0.05) from the control incubations with no diamide or TBHP.

Fig 5. Structures of oxidants utilized in these studies. The chemical structures of diamide (1,1'-azobis(N,N-dimethylformamide)) and TBHP (tert-butyl hydroperoxide) are shown.
Fig. 1
Fig. 4

**A**
Diamide (μM) vs. nmoles/mg protein
- GSH
- GSSG

**B**
TBHP (μM) vs. nmoles/mg protein
- GSH
- GSSG
Fig. 5

Diamide

TBHP