

Activation of Microsomal Glutathione S-Transferase by Peroxynitrite

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ABSTRACT

Peroxynitrite (ONOO⁻) toxicity is associated with protein oxidation and/or tyrosine nitration, usually resulting in inhibition of enzyme activity. We examined the effect of ONOO⁻ on the activity of purified rat liver microsomal glutathione S-transferase (GST) and found that the activity of reduced glutathione (GSH)-free enzyme was increased 4- to 5-fold by 2 mM ONOO⁻; only 15% of this increased activity was reversed by dithiothreitol. Exposure of the microsomal GST to ONOO⁻ resulted in concentration-dependent oxidation of protein sulfhydryl groups, dimer and trimer formation, protein fragmentation, and tyrosine nitration. With the exception of sulfhydryl oxidation, these modifications of the enzyme correlated well

with the increase in enzyme activity. Nitration or acetylation of tyrosine residues of the enzyme using tetranitromethane and *N*-acetylimidazole, respectively, also resulted in increased enzyme activity, providing additional evidence that modification of tyrosine residues can alter catalytic activity. Addition of ONOO⁻-treated microsomal GST to microsomal membrane preparations caused a marked reduction in iron-induced lipid peroxidation, which raises the possibility that this enzyme may act to lessen the degree of membrane damage that would otherwise occur under pathophysiological conditions of increased ONOO⁻ formation.

Glutathione S-transferases (GSTs) play an important role in the detoxification of numerous carcinogenic, mutagenic, toxic, and pharmacologically active compounds (Chasseaud, 1979). The microsomal GST is distinct from its cytosolic counterparts by many criteria (molecular weight, amino acid sequence, immunological properties, subunit composition) (Morgenstern et al., 1982; DeJong et al., 1988; Lundqvist et al., 1992). The microsomal GST contains only one cysteine residue per subunit (Cys49), and enzyme activity can be increased by sulfhydryl modifying reagents such as *N*-ethylmaleimide (NEM), oxidized glutathione, 5,5'-dinitro-bis(2-nitrobenzoic acid) (DTNB), nitric oxide (NO), and *S*-nitroso-glutathione (GSNO) (Morgenstern et al., 1979; Morgenstern et al., 1980; Sies et al., 1998; Ji et al., 2002), as well as by limited proteolysis (Morgenstern et al., 1989), radiation (Boyer et al., 1986) and heating (Aniya, 1989). The activation of the enzyme by reactive oxygen species such as H₂O₂ and superoxide anion is thought to be mediated by *S*-glutathiolation or polymer formation (Aniya and Anders, 1989; 1992), although others have failed to demonstrate activation of the enzyme by H₂O₂ or by superoxide generating systems (Lundqvist and Morgenstern, 1992). The microsomal GST has been

localized to the endoplasmic reticulum, the outer mitochondrial membrane, and the plasma membrane (Morgenstern et al., 1984; Horbach et al., 1993). The rat microsomal GST has a high degree of sequence similarity to the human microsomal GST, and although it is found predominantly in the liver, in both human and rat, the enzyme is extensively distributed in extrahepatic tissues (Otieno et al., 1997; Estonius et al., 1999). In addition to catalyzing typical conjugation reactions with GSH, the microsomal GST possesses selenium-independent glutathione peroxidase activity and catalyzes the reduction of phospholipid hydroperoxides (Mosialou and Morgenstern, 1989), and it has been suggested that activation of the microsomal GST under conditions of oxidative stress may protect cells from oxidative damage (Mosialou and Morgenstern, 1989; Aniya and Anders, 1992).

Nitric oxide is an important regulator of a wide range of physiological and pathological processes. As a biological regulator of many enzyme activities, NO can act either by binding to the heme moiety of hemoproteins or by *S*-nitrosylation/oxidation of sulfhydryl groups (Broillet, 1999). In addition, NO can react with superoxide to produce ONOO⁻ (Pryor and Squadrito, 1995; Beckman and Koppenol, 1996). This highly reactive nitrogen-oxygen species is produced in diverse inflammatory and pathological processes (Patel et al., 1999). Besides participating in tyrosine nitration reactions, ONOO⁻

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ABBREVIATIONS: GST, glutathione S-transferase; GSH, reduced glutathione; NEM, *N*-ethylmaleimide; DTNB, 5,5'-dinitro-bis(2-nitrobenzoic acid); PAGE, polyacrylamide gel electrophoresis; CDNB 1-chloro-2,4-dinitrobenzene; DTPA, diethylenetriaminepentaacetic acid; DTT, dithiothreitol; TBARS, thiobarbituric acid-like reactive substances; TNM, tetranitromethane.

can directly oxidize sulfhydryl groups and can mediate one- or two-electron oxidation reactions with various biological target molecules (Ducrocq et al., 1999). The microsomal GST contains seven tyrosine residues, and in site-directed mutagenesis studies, certain tyrosine to phenylalanine mutants exhibit altered enzymatic activity (Weinander et al., 1997). This raises the possibility that modification of tyrosine residues in the microsomal GST (e.g., by tyrosine nitration) may affect enzyme activity. In addition, because it has been suggested that activation of microsomal GST serves as an antioxidant defense mechanism, it was of interest to determine whether microsomal GST activity could also be regulated by nitrosative stress. Accordingly, we assessed the effect of ONOO⁻ on the activity of purified rat liver microsomal GST.

Experimental Procedures

Materials. Hydroxyapatite, CM-Sepharose, thiobarbituric acid, *N*-acetylhydrazole, 1-chloro-2,4-dinitrobenzene (CDNB), cumene hydroperoxide, DTNB, diethylenetriaminepentaacetic acid (DTPA), dithiothreitol (DTT), GSH, GSH reductase, H₂O₂, NEM, tetranitromethane (TNM), Triton X-100, ADP, and NADPH were purchased from Sigma (St. Louis, MO). Manganese (IV) dioxide was from Aldrich (Milwaukee, WI). Sodium nitrite was from BDH Inc. (Toronto, ON, Canada). Monoclonal anti-nitrotyrosine antibody was from Cayman Chemical Company (Ann Arbor, MI), and horseradish peroxidase-linked goat anti-mouse IgG was obtained from Bio-Rad (Mississauga, ON, Canada). Chemiluminescence reagents were from Kirkegaard and Perry Laboratories (Gaithersburg, MA). All other chemicals were of reagent grade and were obtained from a variety of commercial sources.

Purification of Microsomal GST. Male Sprague-Dawley rats (250–300 g) were fasted overnight, and hepatic microsomes were prepared as described previously (Ji et al., 1996). To remove cytosolic contamination, the microsomes were washed twice with 100 mM Tris-HCl, pH 7.4. Microsomal GST was purified according to Morgenstern and DePierre (1983). Briefly, microsomes (1.3 g of protein) were solubilized with 2.5% Triton X-100, loaded onto a hydroxyapatite column (2.5 × 19 cm), and eluted with a linear gradient of 0.01 to 0.3 M potassium phosphate in buffer A (10 mM potassium phosphate, pH 7.0, 1.0 mM GSH, 0.1 mM EDTA, 1% Triton X-100, and 20% glycerol). Fractions containing NEM-activated GST activity were pooled and dialyzed for 48 h against three changes of 2.0 liters of buffer A. The dialyzed sample was applied to a CM-Sepharose column (1.5 × 9 cm) and eluted with a linear gradient of 0 to 0.2 M KCl in buffer A. Fractions containing NEM-activated GST activity were run on a 15% SDS-PAGE gel. Those fractions without other protein contamination (as assessed by Coomassie Blue staining) were pooled and stored at -70°C. Before experiments, GSH was removed by dialysis of the enzyme preparation (maximum volume, 0.4 ml) for 48 h against three changes of 100 ml of buffer A minus GSH using a System 500 Microdialyzer (Pierce, Rockford, IL). Microsomal GST activity was determined by the spectrophotometric method of Habig et al. (1974). Samples (1.0 ml) contained 100 mM potassium phosphate, pH 6.5, 0.5% Triton X-100, 1 mM GSH, and 1 mM CDNB at 25°C. Enzyme activation by NEM was assessed after incubation of enzyme in 100 mM potassium phosphate, pH 7.0, for 1 min at room temperature with 1.0 mM NEM as described previously (Ji et al., 1996). For kinetic studies, enzyme activities were assayed with constant CDNB concentration (1.0 mM) and varying GSH concentrations (0.1–1.0 mM). The GSH peroxidase activity of microsomal GST was determined (Redd et al., 1981), using cumene hydroperoxide as substrate. Samples (1 ml) contained 1.0 mM GSH, 0.2 mM NADPH, 1.0 U glutathione reductase, 100 mM potassium phosphate, pH 7.0, 0.5% Triton X-100, and 10 µg of purified microsomal GST that had been exposed or not to 2.0 mM ONOO⁻ for 10 s at room

temperature. Samples were preincubated at 37°C for 2 min and the reaction was initiated by the addition of 1.2 mM cumene hydroperoxide. NADPH oxidation was monitored at 340 nm at 37°C.

Preparation of ONOO⁻ and Treatment of Microsomal GST. The ONOO⁻ was synthesized from acidified nitrite and H₂O₂ as described by Beckman et al., (1994) and stored at -70°C. The concentration of ONOO⁻ was determined spectrophotometrically at 302 nm ($\epsilon_{302} = 1670 \text{ M}^{-1}\text{cm}^{-1}$) at the time of synthesis and again before each experiment. The H₂O₂ contamination of ONOO⁻ solutions was removed by manganese dioxide chromatography (Beckman et al., 1994). Microsomes (200–400 µg protein) and purified enzyme (20–80 µg/ml) in 100 mM potassium phosphate, pH 7.0, containing 100 µM DTPA were exposed to ONOO⁻ at room temperature at the concentrations and for the times indicated. ONOO⁻ was added to enzyme preparation as a small volume during vigorous mixing. The reaction was terminated by dilution of the sample into the reaction mixture used for the determination of enzyme activity. To control for the potential effect of nitrite and nitrate that would be formed during the incubation of ONOO⁻, ONOO⁻ was allowed to decompose in phosphate buffer for 10 min at room temperature before the addition of microsomes or microsomal GST. The decomposition of ONOO⁻ was verified by measuring the absorbance at both 302 (for ONOO⁻) and 420 nm (for H₂O₂). The pH of the incubation mixtures was monitored to ensure that the addition of alkaline solutions of ONOO⁻ or decomposed ONOO⁻ did not alter the final pH of the reaction mixture. In other experiments, enzyme was incubated for 15 min at room temperature with TNM at either pH 6 or 8 or with freshly prepared *N*-acetylhydrazole at room temperature for 20 min.

Analysis of Sulfhydryl and Nitrotyrosine Content. The reduced sulfhydryl group content of microsomal GST was determined by the method of Ellman (Riddles et al., 1983). After treatment with various concentrations of ONOO⁻ or TNM, 5 µM microsomal GST was denatured with 1% SDS and incubated with 200 µM DTNB at room temperature for 90 min. The absorbance at 412 nm was determined and the concentration of sulfhydryl groups calculated using $\epsilon_{412} = 13,600 \text{ M}^{-1}\text{cm}^{-1}$. The nitrotyrosine content of microsomal GST was calculated by the increase in absorbance at 430 nm ($\epsilon_{430} = 4400 \text{ M}^{-1}\text{cm}^{-1}$) according to Crow and Ischiropoulos (1996).

SDS-PAGE and Immunoblot Analysis. After treatment with ONOO⁻ or TNM, microsomal GST was resolved on a 15% SDS-PAGE gel under nonreducing conditions. Dimer/trimer isoforms were visualized using Coomassie Blue staining. For detection of nitrotyrosine, proteins were transferred electrophoretically to polyvinylidene difluoride membranes and incubated with a 3-nitrotyrosine-specific antibody. The immunoreactive protein bands were visualized by enhanced chemiluminescence. Relative quantitation of dimer and trimer formation was performed using Corel PHOTO-PAINT software (version 8, Corel Corp., Ottawa, ON, Canada) after scanning of gels with a desktop scanner.

Effect of Cys49 Oxidation by H₂O₂ on Enzyme Activation by ONOO⁻. The extent of sulfhydryl oxidation by H₂O₂ was determined by monitoring the loss in NEM-mediated enzyme activation. Enzyme (20 µg/ml in 100 mM potassium phosphate, pH 7.0) was incubated with various concentrations of H₂O₂ for 10 min at 37°C followed by 1 mM NEM for 1 min at room temperature and enzyme activity assessed. Enzyme activation by H₂O₂ in the absence of NEM was also assessed. Near-maximal loss of NEM-mediated enzyme activation occurred at 20 mM H₂O₂, and this concentration of H₂O₂ was used in subsequent experiments. Enzyme (280 µg/ml) was incubated with H₂O₂ for 10 min at 37°C and then was washed with 100 mM potassium phosphate buffer, pH 7.0, containing 100 µM DTPA, using a Microcon centrifugal filter device (Millipore, Bedford, MA), until the theoretical concentration of H₂O₂ was reduced to less than 5 mM. Enzyme was then incubated at room temperature with 1 mM NEM for 1 min, 2 mM ONOO⁻ for 10 s, or 2 mM ONOO⁻ for 10 s followed by 5 mM DTT for 20 min, and enzyme activity was then assessed.

Determination of Lipid Peroxidation in Microsomes. Lipid peroxidation was initiated by adding NADPH (0.3 mM) and Fe^{3+} -ADP (6 μM –2 mM) to freshly prepared rat hepatic microsomes (0.5 mg/ml protein) in 50 mM Tris-HCl, and 0.14 M NaCl, pH 7.4 at 37°C, as described previously (Burk, 1983), and thiobarbituric acid-like reactive substances (TBARS) were determined spectrophotometrically (Aust, 1985) at various times after the initiation of lipid peroxidation. Some incubations were performed in the presence of 0.1 mM GSH. In others, purified microsomal GST, either untreated or treated with 2 mM ONOO^- , was added to the incubation mixture before initiation of iron-induced lipid peroxidation. The amount of purified microsomal GST added to the hepatic microsomes was approximately 25% of that present in the microsomes, based on an abundance of microsomal GST of 3% of microsomal protein (Morgens-tern et al., 1984).

Results

Effect of ONOO^- on Microsomal GST Activity. Exposure of hepatic microsomes or purified rat hepatic microsomal GST to ONOO^- resulted in a marked increase in enzyme activity. Maximal activation of the enzyme occurred within 5 s at room temperature (Figs. 1A and 2A). Prolonging the

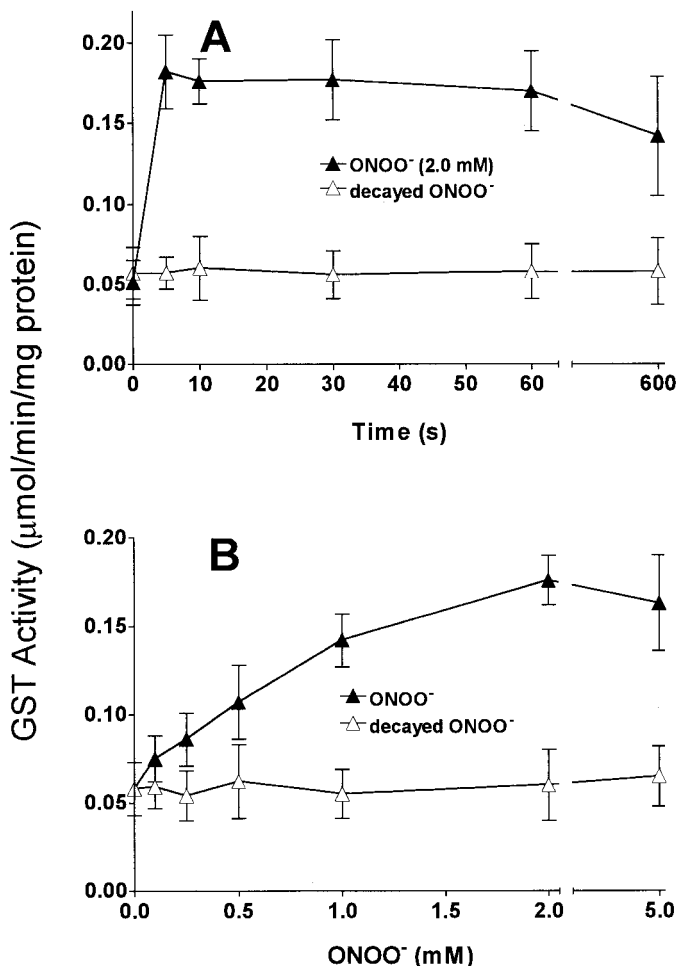


Fig. 1. Effect of ONOO^- on GST activity in rat liver microsomes. Microsomes (0.2–0.5 mg protein) were incubated with 2.0 mM ONOO^- (▲) or decayed ONOO^- (△) for the times indicated (A) or were incubated for 10 s at the concentrations indicated (B). Data points represent the mean \pm S.D. ($n = 3$). GST activity was significantly increased by ONOO^- compared with decayed ONOO^- for all comparisons except 0.1 mM ONOO^- . ($p < 0.05$, Student's t test for unpaired data).

incubation time to 1 min did not result in further activation of the enzyme. However, enzyme activity was decreased about 15% when the enzyme was treated with 2 mM ONOO^- for 10 min (Figs. 1A and 2A) and in subsequent experiments, an incubation time of 10 s was chosen. The activation of microsomal GST by ONOO^- in microsomes or with the purified enzyme occurred in a concentration-dependent manner, with an EC_{50} value for enzyme activation of about 0.25 mM under our experimental conditions (Figs. 1B and 2B). The maximal increase in activity of the purified enzyme was about 4.3-fold using 2 mM ONOO^- , whereas neither decayed ONOO^- nor 2 mM H_2O_2 had any effect on enzyme activity. In kinetic studies (Table 1), treatment of the purified enzyme with ONOO^- resulted in about a 7-fold increase in turnover (k_{cat}) and about a 3-fold increase in enzyme efficiency (k_{cat}/K_m).

ONOO^- -Induced Polymer Formation. Purified microsomal GST was incubated with 2 mM ONOO^- followed by SDS-PAGE under nonreducing conditions (Fig. 3A). Al-

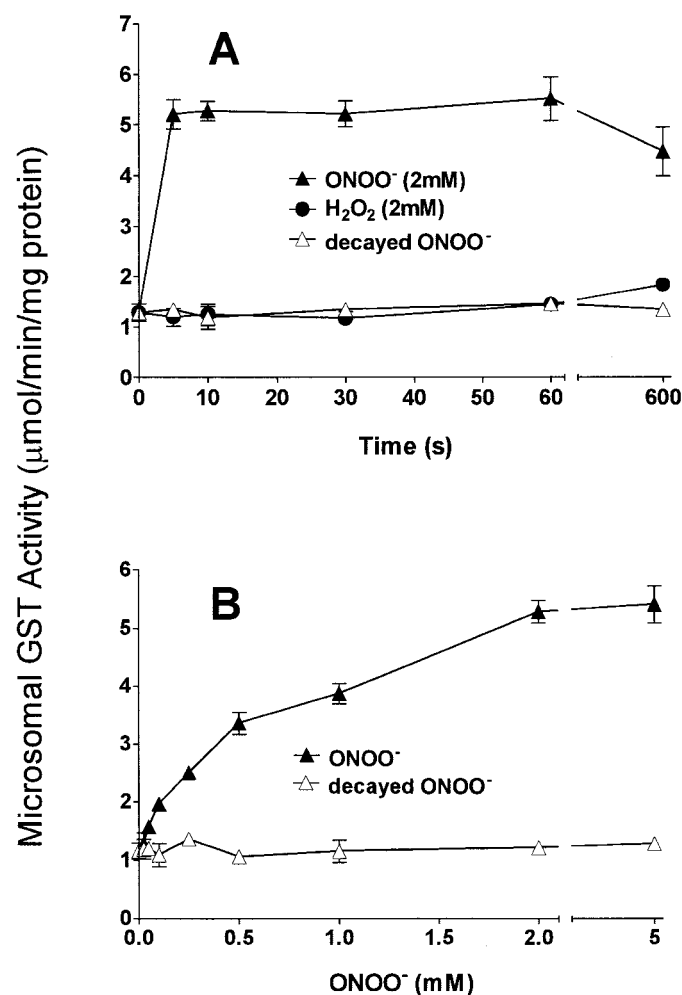


Fig. 2. Effect of ONOO^- on the activity of purified microsomal GST. Purified enzyme (20 $\mu\text{g}/\text{ml}$) was incubated with 2.0 mM ONOO^- (▲), 2.0 mM H_2O_2 (●), or decayed ONOO^- (△) for the times indicated (A) or was incubated for 10 s at the concentrations indicated (B). Data points represent the mean \pm S.D. ($n = 3$). In A, GST activity was significantly increased by ONOO^- compared with the other two groups for all comparisons ($p < 0.001$, one-way ANOVA and Newman-Keuls post hoc test). In B, GST activity was significantly increased by ONOO^- compared with decayed ONOO^- for all comparisons except 0.025 mM ONOO^- ($p < 0.01$, Student's t test for unpaired data).

though the majority of the protein was detected as the monomeric form (about 17 kDa) two other protein bands, with higher molecular masses of about 31 and 45 kDa, were observed, suggesting dimer and trimer formation. In contrast, polymer formation did not occur during incubation of the enzyme with H_2O_2 or decayed $ONOO^-$ (Fig. 3A). Dimer and trimer formation increased with increasing $ONOO^-$ concentration (Fig. 3B), and at 2 mM $ONOO^-$ about 4 and 1% of the total protein was in the dimeric and trimeric form, respectively. In addition, incubation of the enzyme with DTT subsequent to $ONOO^-$ treatment resulted in a partial loss of dimer/trimer formation, suggesting that a portion of dimer/trimer formation by $ONOO^-$ was caused by disulfide bond formation.

Effect of $ONOO^-$ on Free Sulfhydryl Content of Microsomal GST. We quantitated the free sulfhydryl content of microsomal GST using DTNB and found that incubation of microsomal GST with $ONOO^-$ resulted in a concentration-dependent loss of free sulfhydryl groups, with an EC_{50} value of about 0.04 mM (Fig. 4A). At 0.2 mM $ONOO^-$ there was an ~85% reduction in free sulfhydryl content. However, comparison of the concentration dependence of enzyme activation by $ONOO^-$ (Fig. 2B) with the loss of free sulfhydryl groups by $ONOO^-$ (Fig. 4A) indicated that oxidation of Cys49 did not parallel the increase in enzyme activity, suggesting that S-oxidation of Cys49 alone is not responsible for enzyme activation by $ONOO^-$.

Effect of $ONOO^-$ on Tyrosine Nitration and Protein Fragmentation of Microsomal GST. Tyrosine nitration after treatment with $ONOO^-$ was assessed both by measuring the increase in absorbance at 430 nm (Crow and Ischiropoulos, 1996) and by immunoblot analysis using a 3-nitrotyrosine-specific antibody. Tyrosine nitration of the 17-kDa microsomal GST monomer occurred in a concentration-dependent manner (Fig. 4B), with maximal nitration of approximately 60% of the tyrosine residues of the protein after exposure to 2 mM $ONOO^-$. The EC_{50} of $ONOO^-$ for tyrosine nitration was approximately 0.35 mM (Fig. 4B). Thus tyrosine nitration more closely paralleled enzyme activation by $ONOO^-$, in contrast to the dissociation of EC_{50} values for sulfhydryl oxidation and enzyme activation by $ONOO^-$. In addition to the nitration of the 17-kDa GST monomer, bands of both lower and higher molecular mass were observed. The lower molecular mass bands probably represent protein fragmentation, whereas higher molecular mass bands seemed to be predominantly tyrosine-nitrated GST dimers and trimers. The other high molecular mass bands could represent cross-linking of protein fragments with GST dimers and trimers.

Effect of Tetranitromethane and *N*-Acetylimidazole on Microsomal GST Activity. To further examine the effect of tyrosine residue modification on microsomal GST ac-

tivity, we assessed the effects of TNM, which oxidizes sulfhydryl groups at both pH 6 and 8 but selectively nitrates tyrosine residues at pH 8 (Sokolovsky et al., 1966). This is clearly evident from examination of the nitrotyrosine immunoblots in Fig. 5C, in which purified microsomal GST was exposed to increasing concentration of TNM at either pH. Also evident is the greater degree of tyrosine-nitrated dimer and trimer formation that occurred at pH 8, protein fragmentation, and the partial reduction in dimer/trimer content after treatment with DTT (Fig. 5B). Thus the structural changes to microsomal GST after treatment with TNM and $ONOO^-$ were qualitatively similar (compare Figs. 4B and 5C). Microsomal GST activity was increased after treatment with TNM at either pH, but the profile for activation differed (Fig. 5A). At pH 6, GST activity increased in a concentration-dependent manner (0–20 μ M), with an EC_{50} value of about 7.5 μ M and a maximal GST activity of 4.6 μ mol/min/mg of protein. In contrast, at pH 8, the GST activity increased much more rapidly over a range of 0 to 5 μ M, with an EC_{50} value for activation of about 2.5 μ M and maximal activation of 6.6 μ mol/min/mg protein, and thereafter declined over the concentration range of 5 to 20 μ M (Fig. 5A). *N*-Acetylimidazole, another tyrosine modifying reagent, was also used to study the effect of tyrosine modification on enzyme activity. *N*-Acetylimidazole increased microsomal GST activity in a dose-dependent manner, with an EC_{50} value of about 5 mM, and maximal activation of approximately 3-fold (Fig. 6). Enzyme activation by *N*-acetylimidazole was not accompanied by protein fragmentation or polymer formation (data not shown). However, like the other tyrosine-modifying reagents, *N*-acetylimidazole also oxidized Cys49 of the enzyme, making it difficult to assess the relative role of tyrosine modification for the increase in enzyme activity caused by this reagent.

Effect of Cys49 Oxidation by H_2O_2 on Enzyme Activation by $ONOO^-$. In this series of experiments, we assessed the effect of sequential modification of Cys49 and tyrosine residues on enzyme activity by exposing the enzyme first to H_2O_2 and then to $ONOO^-$. Incubation of enzyme with H_2O_2 resulted in a concentration-dependent oxidation of Cys49, as evidenced by the loss of NEM-mediated enzyme activation when added subsequent to H_2O_2 treatment (Fig. 7). However, under our experimental conditions, H_2O_2 treatment resulted in a negligible increase in enzyme activity (Fig. 7), indicating that modification of Cys49 to higher oxidation states alone is insufficient to support an increase in enzyme activity. When the enzyme was treated with $ONOO^-$ subsequent to oxidation of Cys49 by H_2O_2 , an increase in enzyme activity was observed, approaching that obtained after treatment of the enzyme with $ONOO^-$ alone (Fig. 8). This increase in activity was not altered by DTT.

Protective Effect of Microsomal GST on Iron-Induced Lipid Peroxidation. The microsomal GST possesses selenium-independent GSH peroxidase activity, and a final series of experiments was performed to assess whether $ONOO^-$ -treated microsomal GST exhibited protective effects against iron-induced lipid peroxidation. Using cumene hydroperoxide as substrate, we first assessed whether treatment of purified microsomal GST with $ONOO^-$ increased the GSH peroxidase activity of the enzyme. As seen in Fig. 9, $ONOO^-$ treatment resulted in about a 3.5-fold increase in peroxidase activity, an increase similar to that seen using CDNB as substrate. We then assessed the effect of $ONOO^-$ -

TABLE 1

Kinetic studies of microsomal GST activation by $ONOO^-$

Purified enzyme (20 μ g/ml) was incubated with 2.0 mM $ONOO^-$ for 10 s at room temperature. GST activity was measured using 1.0 mM CDNB and varying concentrations of GSH (0.1–1.0 mM) (values represent the mean \pm S.D., $n = 6$).

Condition	K_{cat} s^{-1}	K_m mM	K_{cat}/K_m $s^{-1} mM^{-1}$
Control	1.6 ± 0.1	2.6 ± 0.2	0.59
$ONOO^-$	$11 \pm 2.5^{**}$	$12 \pm 3.2^{**}$	1.67

** , $P < 0.001$ vs control, Student's t test for unpaired data.

treated microsomal GST on iron-induced lipid peroxidation of hepatic microsomes. In control microsomes, half-maximal TBARS formation occurred after 6 min, with a maximal TBARS formation of about 40 nmol/mg of protein occurring 20 min after initiation (Fig. 10). As reported by others (Mossialou and Morgenstern, 1989), addition of GSH to microsomes before initiation of lipid peroxidation resulted in a

protective effect, presumably by providing substrate for the endogenous microsomal GST present in the microsomal preparation. This was manifested by a significant decrease in the maximal TBARS formation and an increase in the time to half-maximal appearance of TBARS. We then coincubated microsomes with an amount of purified microsomal GST equal to approximately 25% of that present in the microso-

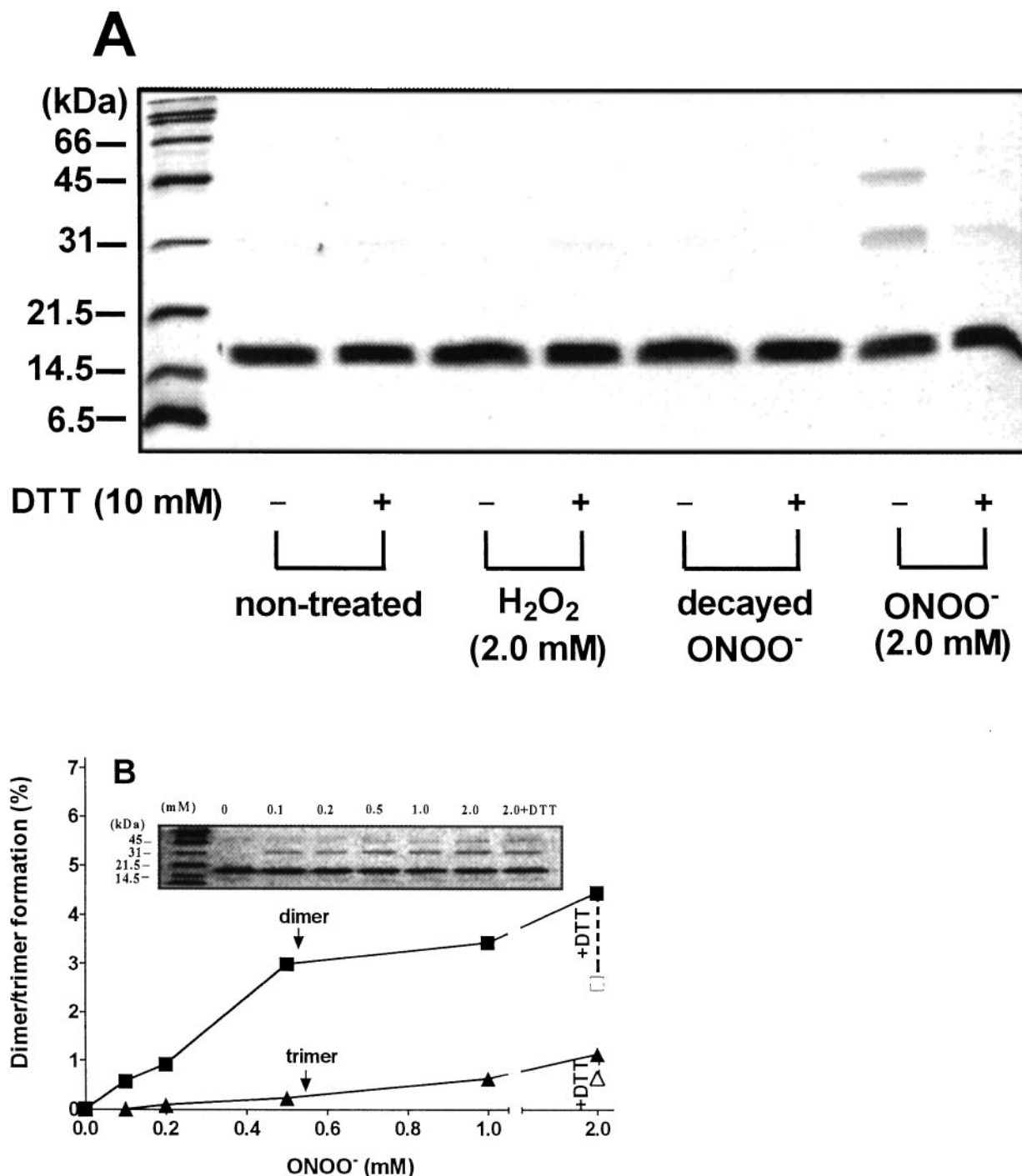


Fig. 3. Effect of ONOO^- on microsomal GST dimer and trimer formation. A, purified enzyme (40 $\mu\text{g}/\text{ml}$) was incubated with 2.0 mM ONOO^- , decomposed ONOO^- , or 2.0 mM H_2O_2 for 10 s at room temperature. A portion of each sample was then incubated with 10 mM DTT for an additional 20 min. B, purified enzyme (80 $\mu\text{g}/\text{ml}$) was incubated with increasing concentrations of ONOO^- for 10 s at room temperature. Samples were resolved on a 15% SDS-PAGE gel under nonreducing conditions (2.0 μg protein/lane), and protein bands were visualized using Coomassie Blue staining. The relative dimer and trimer formation in (B) was determined using the density of the band of nontreated enzyme as 100%. The reduction in dimer (\square) and trimer (\triangle) formation after treatment with DTT is also shown.

mal preparation. In the presence of GSH, this had only a minimal additional protective effect against iron-induced lipid peroxidation. However, when the purified microsomal GST was first treated with ONOO^- , there was a marked inhibition of TBARS formation (Fig. 10), indicating that ONOO^- -activated enzyme was very effective in preventing iron-induced lipid peroxidation.

Discussion

In addition to participating in the biotransformation of xenobiotics, the GSH peroxidase activity of the microsomal GST catalyzes the reduction of phospholipid hydroperoxides and has been shown to protect membranes from oxidant-induced lipid peroxidative damage (Mosialou and Morgenstern, 1989). Activation of this enzyme by covalent modification of its sole sulfhydryl group (Cys49) is a characteristic that distinguishes the microsomal GST from its cytosolic counterparts. Recently, we found that *S*-nitrosoglutathione and the NO donor 1,1-diethyl-2-hydroxy-2-nitrosohydrazine can activate the enzyme via *S*-nitrosylation of Cys49 (Ji et al., 2002). This implied that alteration of microsomal GST

activity could occur under conditions of nitrosative stress; hence, we wished to investigate whether this enzyme could be a target for ONOO^- .

A powerful oxidant, ONOO^- can react with a range of biological molecules and subsequently cause a number of modifications of cellular structure and function (e.g., oxidation of thiols, tyrosine nitration, lipid peroxidation, inactivation of ion channels, and damage to DNA). There are an increasing number of recent reports describing the inhibitory effect of ONOO^- on the enzymatic activity of a variety of proteins, including manganese superoxide dismutase (MacMillan-Crow et al., 1998), tryptophan hydroxylase (Kuhn and Geddes, 1999), tyrosine hydroxylase (Ara et al., 1998; Kuhn et al., 1999; Blanchard-Fillion et al., 2001; Kuhn et al., 2002), xanthine oxidase (Lee et al., 2000), sarcoplasmic reticulum Ca-ATPase (Viner et al., 1999), creatine kinase (Konorev et al., 1998), alcohol dehydrogenase (Crow et al., 1995), cytosolic GSTs (Wong et al., 2001), protein kinase C (Knapp et al., 2001), GSH reductase (Savvides et al., 2002), endothelial NO synthase (Zou et al., 2002), protein tyrosine phosphatases (Takakura et al., 1999), and cytochrome *c* (Cassina et al.,

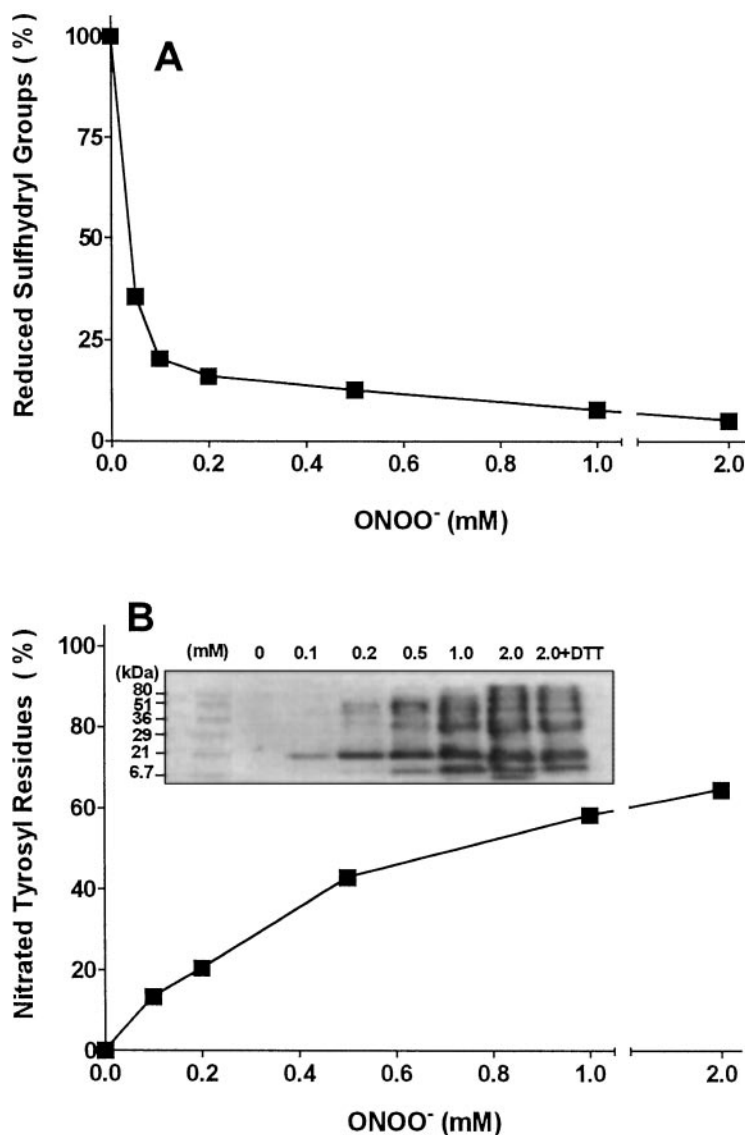


Fig. 4. Structural modifications of microsomal GST by ONOO^- . Purified enzyme (80 $\mu\text{g}/\text{ml}$) was exposed to the indicated concentrations of ONOO^- at room temperature for 10 s, and loss of sulfhydryl groups (A) and tyrosine nitration (B) were assessed as described under *Experimental Procedures*. In B, the degree of tyrosine nitration was expressed relative to the total number of tyrosine residues in the protein. Samples were resolved on a 15% SDS-PAGE gel under nonreducing conditions (2.0 μg protein/lane), and protein bands visualized by immunoblotting using an anti-nitrotyrosine antibody.

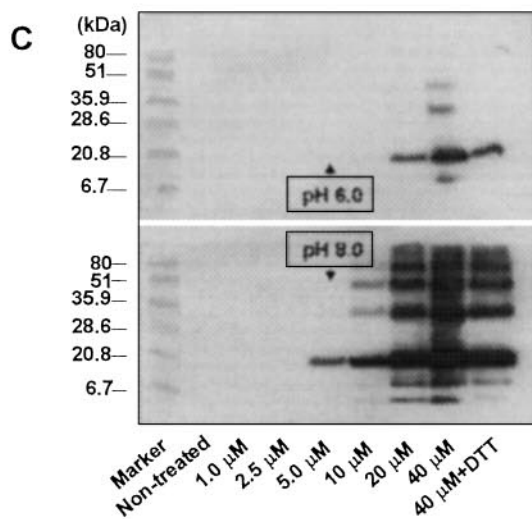
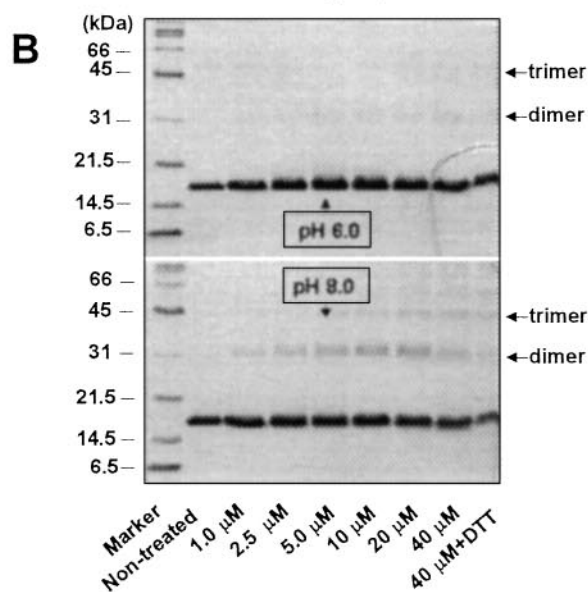
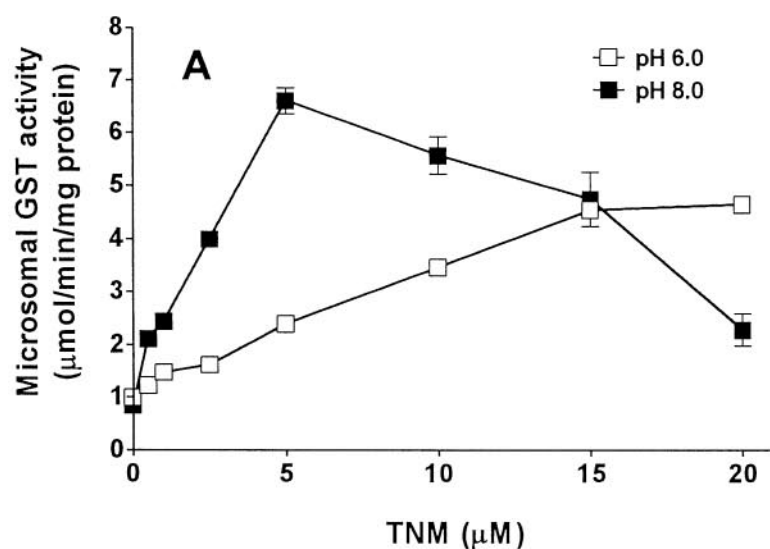


Fig. 5. Effect of TNM on enzyme activity and structural modifications of microsomal GST. A, purified enzyme (20 $\mu\text{g/ml}$) was incubated with the indicated concentrations of TNM at either pH 6.0 (\square) or pH 8.0 (\blacksquare) at room temperature for 15 min, and enzyme activity was assessed. Data points represent the mean \pm S.D. ($n = 3$). B and C, samples were treated as in A and samples were resolved on a 15% SDS-PAGE gel under nonreducing conditions (2.0 μg protein/lane). Protein bands visualized by Coomassie Blue staining (B) or by immunoblotting using an anti-nitrotyrosine antibody (C). In A, GST activity at pH 8.0 was significantly different from that at pH 6.0 at all TNM concentrations except 15 μM ($p < 0.001$, Student's t test for unpaired data).

2000). In most cases, this inhibitory activity has been attributed to nitration of critical tyrosine residues, although in some cases, disruption of active site Zn-thiolate clusters (Crow et al., 1995; Zou et al., 2002) or oxidation of critical sulfhydryl groups has been proposed (Kuhn and Geddes, 1999; Kuhn et al., 1999, 2002; Takakura et al., 1999; Viner et al., 1999). However, in contradistinction to the inhibitory effects of ONOO^- on all of these enzyme activities, ONOO^- markedly stimulated rat liver microsomal GST activity in both hepatic microsomes and in purified enzyme preparations (Figs. 1 and 2). The maximal enzyme activation was 4- to 5-fold, and in kinetic studies, there was a 7-fold increase in turnover and a 3-fold increase in efficiency (Table 1). Although prolonged incubation with higher concentrations of H_2O_2 has been reported to activate microsomal GST (Aniya and Anders, 1992) this has not been a consistent finding (Lundqvist and Morgenstern, 1992). In the present study, the effects of ONOO^- on microsomal GST activity are unlikely to be related to any contaminating H_2O_2 , nitrate, or nitrite

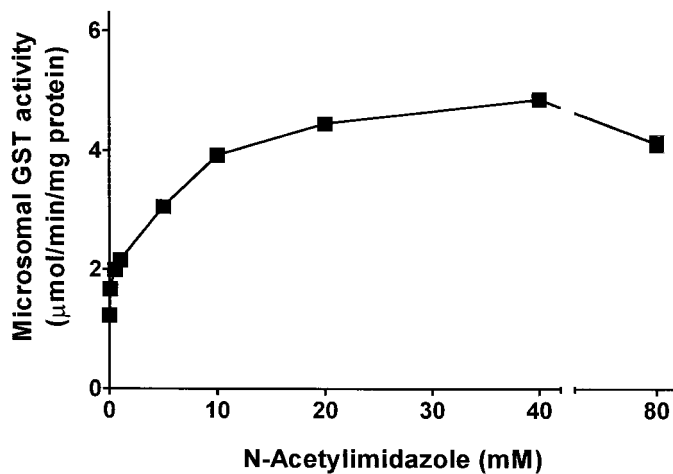


Fig. 6. Effect of *N*-acetylimidazole on microsomal GST activity. Purified enzyme (20 $\mu\text{g}/\text{ml}$) was incubated with the indicated concentrations of *N*-acetylimidazole at room temperature for 20 min, and enzyme activity assessed. Data points represent the mean \pm S.D. ($n = 3$).

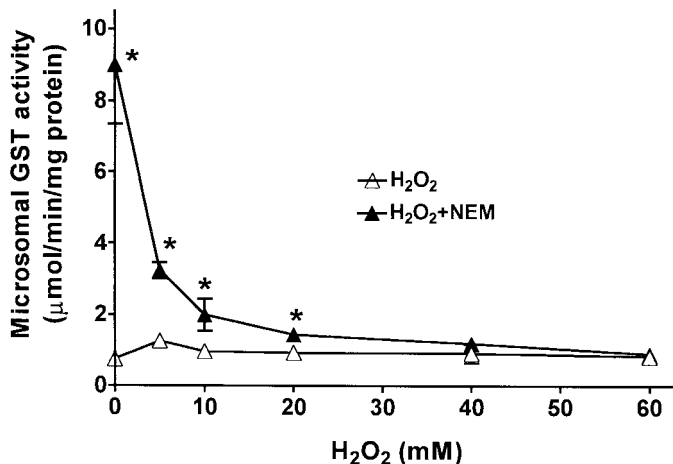


Fig. 7. Effect of H_2O_2 on microsomal GST activity. Purified enzyme (20 $\mu\text{g}/\text{ml}$) was incubated with the indicated concentrations of H_2O_2 at 37°C for 10 min (Δ). In experiments using NEM (\blacktriangle), H_2O_2 -treated enzyme was exposed to 1.0 mM NEM for one additional minute before the assessment of enzyme activity. Data points represent the mean \pm S.D. ($n = 3$). *, $p < 0.05$ versus H_2O_2 , Student's *t* test for unpaired data.

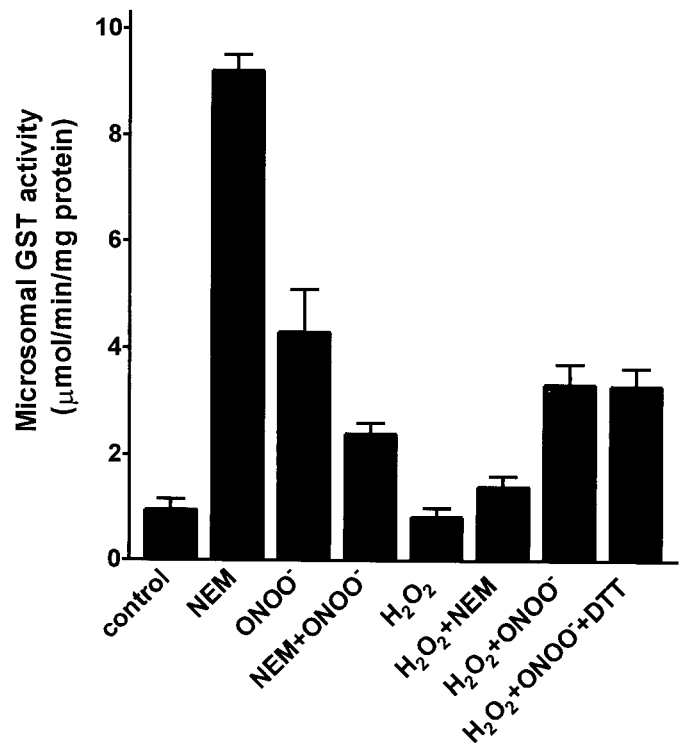


Fig. 8. Comparison of the effect of various modifying reagents on microsomal GST activity. Purified enzyme (20 $\mu\text{g}/\text{ml}$) was incubated with various reagents, either alone or in the order indicated in on the abscissa, and enzyme activity was assessed. The treatments were: NEM, 1 mM for 1 min at room temperature; ONOO^- , 2 mM for 10 s at room temperature; H_2O_2 , 20 mM for 20 min at 37°C ; and DTT, 5 mM at room temperature for 20 min. Data points represent the mean \pm S.D. ($n = 3-4$). All comparisons were significantly different except: control versus $\text{H}_2\text{O}_2 \pm \text{NEM}$, H_2O_2 versus $\text{H}_2\text{O}_2 + \text{NEM}$, and $\text{H}_2\text{O}_2 + \text{ONOO}^-$ versus $\text{H}_2\text{O}_2 + \text{ONOO}^- + \text{DTT}$ ($p < 0.05$, one-way ANOVA and Newman-Keuls post hoc test).

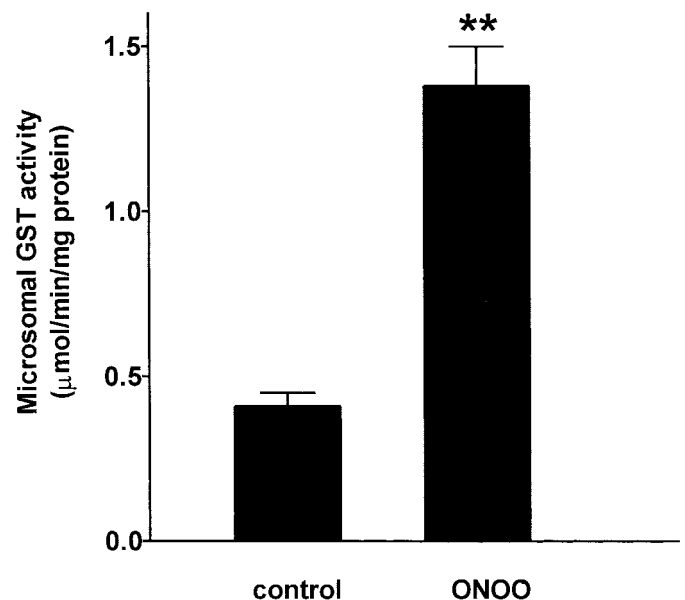


Fig. 9. Effect of ONOO^- on GSH-peroxidase activity of microsomal GST. Purified enzyme (100 $\mu\text{g}/\text{ml}$) was incubated with or without 2.0 mM ONOO^- at room temperature for 10 s and the GSH peroxidase activity determined using cumene hydroperoxide as substrate. Data represent the mean \pm S.D. ($n = 3$). **, $p < 0.001$ versus control, Student's *t* test for unpaired data.

present in the ONOO^- solutions, because neither decomposed solutions of ONOO^- nor incubations using an equal concentration of H_2O_2 (2 mM) had any effect on enzyme activity over the time course examined (Figs. 1 and 2).

Because ONOO^- can modify proteins by different mechanisms (Ischiropoulos and Al-Mehdi, 1995; Ducrocq et al., 1999), we assessed several potential modifications of purified microsomal GST by ONOO^- and attempted to correlate these with the degree of enzyme activation. As shown in Fig. 3, A and B, treatment of microsomal GST with ONOO^- resulted in concentration-dependent dimer and trimer formation, the extent of which correlated with the increase in enzyme activity (Fig. 2B). In a previous study (Aniya and Anders, 1992), treatment of hepatic microsomes with H_2O_2 also resulted in dimer and trimer formation, and this also was associated with an increase in enzyme activity. Dimer and trimer formation was reversed by treatment of microsomes with DTT, concomitant with a decrease in enzyme activity. However, in these studies, in contrast to our own, treatment of the enzyme with H_2O_2 was performed in the presence of GSH; thus, the relative contribution of oligomerization versus *S*-glutathiolation to the increase in enzyme activity could not be ascertained. In the present study, DTT reversed only approximately one third of the polymer formation caused by ONOO^- , and it would seem reasonable to suggest that the remaining two thirds may have been formed through dityrosine cross-linking, because this has been reported for other proteins after exposure to ONOO^- (MacMillan-Crow et al., 1998; Kuhn et al., 1999; Schwemmer et al., 2000; Blanchard-Fillion et al., 2001).

In the native state, the microsomal GST is thought to exist as a homotrimer (Lundqvist et al., 1992), although on both reducing and nonreducing SDS-PAGE gels, the enzyme migrates as the 17-kDa monomer. If covalent oligomerization of the microsomal GST by ONOO^- does contribute to the in-

crease in enzyme activity, then the conformation of the covalently modified enzyme must presumably be different from that of the native trimer, because the latter merely supports basal enzyme activity. Protein fragmentation has been observed after exposure of bovine serum albumin to ONOO^- (Ischiropoulos and Al-Mehdi, 1995), and treatment of the purified microsomal GST enzyme with ONOO^- resulted in the concentration-dependent formation of nitrated protein fragments (Fig. 4B), the extent of which also correlated with the increase in enzyme activity. Because activation of microsomal GST after trypsin cleavage at Lys-4 and Lys-41 is another characteristic of this enzyme (Morgenstern et al., 1989), the ONOO^- -induced protein fragmentation could certainly contribute to the observed increase in enzyme activity.

Oxidation of critical sulfhydryl groups by ONOO^- has been proposed as the mechanism for its inhibitory actions on several enzymes, including tryptophan (Kuhn and Geddes, 1999) and tyrosine (Kuhn et al., 1999) hydroxylases, sarcoplasmic reticulum Ca-ATPase (Viner et al., 1999), and protein tyrosine phosphatases (Takakura et al., 1999), although the relative importance of sulfhydryl oxidation versus tyrosine nitration for inhibition of tyrosine hydroxylase by ONOO^- is controversial (Kuhn et al., 1999; Blanchard-Fillion et al., 2001; Kuhn et al., 2002). For the protein tyrosine phosphatase PTP1B, reaction of ONOO^- with protein thiol groups was rapid, and the IC_{50} value for enzyme inactivation was less than 1 μM (Takakura et al., 1999). In contrast, 40 μM ONOO^- was required to observe any tyrosine nitration of the enzyme, at which point enzyme activity was inhibited by more than 95%. The microsomal GST was also much more susceptible to sulfhydryl oxidation than to tyrosine nitration; exposure to 0.1 mM ONOO^- resulted in an 80% loss of free sulfhydryl groups (Fig. 4A), whereas tyrosine nitration was barely detectable (Fig. 4B). However, in this case, sulfhydryl oxidation did not correlate well with the change in enzyme activity. For example, at 50 μM ONOO^- , enzyme activity was only slightly increased, whereas there was a 65% loss of free sulfhydryl groups (Figs. 2 and 4A). Thus it cannot be assumed that modifications most susceptible to alteration by ONOO^- would necessarily be the basis for the effects of ONOO^- on enzyme activity. The lack of effect of sulfhydryl oxidation per se on GST activity was further exemplified by the experiments using H_2O_2 (Fig. 7), in which enzyme activity was unaltered despite the almost complete oxidation of Cys49 by this reagent.

Tyrosine nitration can inactivate enzymes that depend on tyrosine residues for their activity, and 3-nitrotyrosine has been used as a biological marker to monitor the *in vivo* production of ONOO^- (Crow and Ischiropoulos, 1996; Schwemmer et al., 2000). There is considerable evidence indicating that the phenolic hydroxyl group of a tyrosine residue at the GSH-binding site of cytosolic GSTs plays a critical role in catalysis by stabilizing the nucleophilic thiolate anion of enzyme-bound GSH. In studies using purified mouse hepatic GST μ , this tyrosine residue was preferentially nitrated by ONOO^- . However, tyrosine nitration could not account fully for the inhibitory effect of ONOO^- because only partial nitration of this tyrosine residue occurred at concentrations of ONOO^- that completely inactivated the enzyme (Wong et al., 2001). For the microsomal GST, tyrosine does not seem to play a similar role in the stabilization of the GSH thiolate anion, because in site-directed mutagenesis studies,

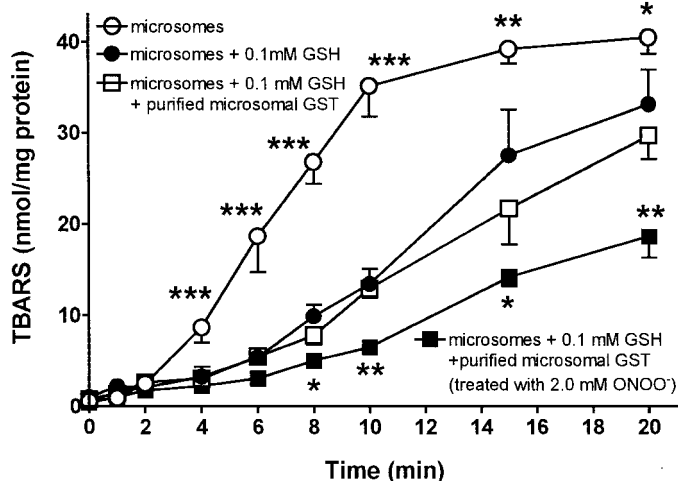


Fig. 10. Effect of addition of ONOO^- -treated microsomal GST on iron-induced lipid peroxidation of rat liver microsomes. Iron-induced lipid peroxidation was assessed by the formation of TBARS at the indicated times in freshly prepared, untreated microsomes (○), or in microsomes incubated in the presence of 0.1 mM GSH (●), 0.1 mM GSH plus purified microsomal GST (□), or 0.1 mM GSH plus purified microsomal GST treated with 2 mM ONOO^- (■). The amount of purified microsomal GST added was equivalent to 25% of that present in the microsomal preparation. Data points represent the mean \pm S.D. ($n = 3$). *, $p < 0.05$ versus all other groups, **, $p < 0.01$ versus all other groups, ***, $p < 0.001$ versus all other groups (one-way ANOVA and Newman-Keuls post hoc test).

tyrosine-to-phenylalanine substitutions did not result in significant decreases in catalytic activity (Weinander et al., 1997). In fact, the Y137F mutant had almost 4-fold greater activity than the wild type enzyme, suggesting a role for this tyrosine residue in stabilizing the unactivated conformation of the enzyme (Weinander et al., 1997).

Our data showed that ONOO⁻ caused tyrosine nitration of the microsomal GST in a dose-dependent manner (Fig. 4B) that paralleled quite closely the increase in enzyme activity (Figs. 2B and 4B). The relative importance of tyrosine nitration versus sulfhydryl oxidation in mediating the increase in enzyme activity by ONOO⁻ was assessed by several different means. TNM is a reagent that oxidizes sulfhydryl groups at pH 6 and pH 8, but selectively nitrates tyrosine residues at pH 8, and this pH-dependent reactivity has been used to differentiate between oxidation and nitration reactions (Sokolovsky et al., 1966; MacMillan-Crow et al., 1998; Kuhn et al., 1999; Kuhn and Geddes, 1999). Overall, the structural modifications caused by TNM were qualitatively the same as ONOO⁻. Although the greater sensitivity of the enzyme for activation by TNM at pH 8 compared with pH 6 (Fig. 5A) would argue in favor of a significant role for tyrosine nitration in enzyme activation, we also found that sulfhydryl oxidation was more extensive at pH 8 than pH 6; incubation of enzyme with 5 μ M TNM at pH 8 resulted in a 68% loss of free sulfhydryl groups, whereas the loss after incubation with 20 μ M TNM at pH 6 was only 17% (data not shown). However, the finding that maximal activation of the enzyme by TNM occurred with 5 μ M TNM at pH 8 (Fig. 5A), and that an equivalent degree of sulfhydryl oxidation occurred with low ONOO⁻ concentration (Fig. 4A) with little increase in enzyme activity, would suggest that tyrosine nitration plays a more significant role in activation of the enzyme. The increase in enzyme activity after exposure to the tyrosine-modifying reagent *N*-acetylimidazole (Fig. 6) provides additional evidence that covalent modification of tyrosine residues of the enzyme can alter its catalytic properties. It is noteworthy that activation of the microsomal GST by *N*-acetylimidazole was not accompanied by protein fragmentation or polymer formation, suggesting that tyrosine modification alone is sufficient to mediate an increase in enzyme activity.

We further evaluated the relative role of sulfhydryl oxidation and tyrosine nitration for mediating increases in microsomal GST activity by assessing the effect of sequential modification of Cys49 and tyrosine residues. The oxidation of Cys49 by H₂O₂ was monitored by the loss of NEM-mediated increases in enzyme activity (because alkylation by NEM requires reduced sulfhydryl groups), and it is clear from the data in Fig. 7 that conversion of the Cys49 sulfur to higher oxidation states by H₂O₂ was not sufficient to cause an increase in enzyme activity. When the oxidized enzyme was subsequently exposed to ONOO⁻, an increase in activity did occur, suggesting that modifications other than sulfhydryl oxidation alone are responsible for activation by ONOO⁻ (Fig. 8). Taken together, the data obtained provide strong correlative evidence for an important role of tyrosine nitration in enzyme activation by ONOO⁻. However, because the enzyme is far more susceptible to sulfhydryl oxidation by ONOO⁻, it was not possible to assess the effect of tyrosine nitration in the absence of sulfhydryl oxidation; therefore, it

cannot be ruled out that both modifications are required for activation.

The critical tyrosine residues responsible for mediating the increase in GST activity are likely to be situated in hydrophobic domains of the protein, because TNM (a hydrophobic reagent) was almost 100-fold more potent than ONOO⁻. It would also seem that more extensive nitration by TNM can reverse the activation seen at lower TNM concentration (Fig. 5A). Of interest is that ONOO⁻ inhibited the marked increase in activity observed after alkylation of Cys49 with NEM. Treatment with NEM alone caused a 13-fold increase in activity, but when the enzyme was subsequently exposed to ONOO⁻, enzyme activation was less than that seen with ONOO⁻ alone (Fig. 8). This would suggest that ONOO⁻ causes conformational changes to the enzyme that are quite different from those caused by alkylation of Cys49. The three dimensional crystal structure of the microsomal GST has yet to be reported, and this would obviously aid in predicting how alteration of particular tyrosine residues might change the conformation of the active site of the protein.

The glutathione peroxidase activity of the microsomal GST catalyzes the reduction of phospholipid hydroperoxides and has been shown to protect membranes from oxidant-induced lipid peroxidative damage (Mosialou and Morgenstern, 1989). Because ONOO⁻ itself can initiate lipid peroxidation, we wished to determine whether the extent of lipid peroxidative damage might be lessened by concomitant activation of microsomal GST by ONOO⁻. To avoid difficulties in interpretation of data that would arise from merely treating microsomal membranes with ONOO⁻, we used iron-induced lipid peroxidation as the model of peroxidative damage, and assessed the effect of adding back purified microsomal GST that had been exposed to ONOO⁻. The significant decrease in the maximal TBARS formation that occurred with the addition of GSH to microsomes before the initiation of lipid peroxidation suggests that the endogenous microsomal GST present in the microsomal membrane preparation has a protective effect (Mosialou and Morgenstern, 1989; Fig. 10). However, this protective effect was significantly greater in membranes to which a relatively small amount (25% of that present in the microsomal membrane preparation) of ONOO⁻-treated microsomal GST had been added (Fig. 10). This suggests that under conditions of exposure to reactive nitrogen species, concomitant activation of the microsomal GST could function to limit the degree of lipid peroxidative damage that would otherwise occur. The activation of the microsomal GST by ONOO⁻ and the cellular protection this may afford becomes more relevant when one considers that other enzymes [e.g., superoxide dismutase (MacMillan-Crow et al., 1998), cytosolic GSTs (Wong et al., 2001), and GSH peroxidase (Savvides et al., 2002)] involved in defense against oxidative stress are inactivated by ONOO⁻.

In summary, we have found that, in contrast to all other enzymes reported so far, the microsomal GST is activated on exposure to ONOO⁻, and nitration of tyrosine residues in the protein rather than sulfhydryl oxidation seems to be the more important modification for mediating this effect. The activation of microsomal GST by ONOO⁻ may play an important role in limiting the extent of oxidative tissue injury when other cellular antioxidant defense mechanisms are compromised under pathophysiological conditions of excessive ONOO⁻ formation.

References

- Aniya Y (1989) Activation of liver microsomal glutathione S-transferase activity by heating. *J Pharmacobiodyn* **12**:235–240.
- Aniya Y and Anders MW (1989) Activation of rat liver microsomal glutathione S-transferase by reduced oxygen species. *J Biol Chem* **264**:1998–2002.
- Aniya Y and Anders MW (1992) Activation of rat liver microsomal glutathione S-transferase by hydrogen peroxide: role for protein-dimer formation. *Arch Biochem Biophys* **296**:611–616.
- Ara J, Przedborski S, Naini AB, Jackson-Lewis V, Trifiletti RR, Horwitz J, and Ischiropoulos H (1998) Inactivation of tyrosine hydroxylase by nitration following exposure to peroxynitrite and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). *Proc Natl Acad Sci USA* **95**:7659–7663.
- Aust SD (1985) Lipid peroxidation, in *CRC Handbook of Methods for Oxygen Radical Research* (Greenwald RA ed) pp 203–207, CRC Press, Boca Raton.
- Beckman JS, Chen J, Ischiropoulos H, and Crow JP (1994) Oxidative chemistry of peroxynitrite. *Methods Enzymol* **233**:229–240.
- Beckman JS and Koppenol WH (1996) Nitric oxide, superoxide, and peroxynitrite: the good, the bad and ugly. *Am J Physiol* **271**:C1424–C1437.
- Blanchard-Fillion B, Souza JM, Friel T, Jiang GC, Vrana K, Sharov V, Barron L, Schoneich C, Quijano C, Alvarez B, et al. (2001) Nitration and inactivation of tyrosine hydroxylase by peroxynitrite. *J Biol Chem* **276**:46017–46023.
- Boyer TD, Vessey DA, and Kempner E (1986) Radiation inactivation of microsomal glutathione S-transferase. *J Biol Chem* **261**:16963–16968.
- Broillet M-C (1999) S-Nitrosylation of proteins. *Cell Mol Life Sci* **55**:1036–1042.
- Burk RF (1983) Glutathione-dependent protection by rat liver microsomal protein against lipid peroxidation. *Biochim Biophys Acta* **757**:21–28.
- Cassina AM, Hodara R, Souza JM, Thomson L, Castro L, Ischiropoulos H, Freeman BA, and Radi R (2000) Cytochrome c nitration by peroxynitrite. *J Biol Chem* **275**:21409–21415.
- Chasseaud LF (1979) The role of glutathione and glutathione S-transferases in the metabolism of chemical carcinogens and other electrophilic agents. *Adv Cancer Res* **29**:175–274.
- Crow JP, Beckman JS, and McCord JM (1995) Sensitivity of the essential zinc-thiolate moiety of yeast alcohol dehydrogenase to hypochlorite and peroxynitrite. *Biochemistry* **34**:3544–3552.
- Crow JP and Ischiropoulos H (1996) Detection and quantitation of nitrotyrosine residues in proteins: in vivo marker of peroxynitrite. *Methods Enzymol* **269**:185–194.
- DeJong J, Morgenstern R, Jörnval H, DePierre JW, and Tu C-PD (1988) Gene expression of rat and human microsomal glutathione S-transferases. *J Biol Chem* **263**:8430–8436.
- Ducrocq C, Blanchard B, Pignatelli B, and Ohshima H (1999) Peroxynitrite: an endogenous oxidizing and nitrating agent. *Cell Mol Life Sci* **55**:1068–1077.
- Estonius M, Forsberg L, Danielsson O, Weinander R, Kelner MJ, and Morgenstern R (1999) Distribution of microsomal glutathione transferase 1 in mammalian tissues. A predominant alternate first exon in human tissues. *Eur J Biochem* **260**:409–413.
- Habig WH, Pabst MJ, and Jacoby WB (1974) Glutathione S-transferases: the first enzymatic step in mercapturic acid formation. *J Biol Chem* **249**:7130–7139.
- Horbach ME, Sies H, and Akerboom TPM (1993) Identification of a hepatic plasma membrane glutathione S-transferase activated by N-ethylmaleimide. *Biochim Biophys Acta* **1148**:61–66.
- Ischiropoulos H and Al-Mehdi AB (1995) Peroxynitrite-mediated oxidative protein modification. *FEBS Lett* **364**:279–282.
- Ji Y, Akerboom TPM, and Sies H (1996) Microsomal formation of S-nitrosoglutathione from organic nitrates: possible role of membrane-bound glutathione transferase. *Biochem J* **313**:377–380.
- Ji Y, Toader V, and Bennett BM (2002) Regulation of microsomal and cytosolic glutathione S-transferase activities by S-nitrosylation. *Biochem Pharmacol* **63**:1397–1404.
- Konorev EA, Hogg N, and Kalyanaraman B (1998) Rapid and irreversible inhibition of creatine kinase by peroxynitrite. *FEBS Lett* **427**:171–174.
- Knapp LT, Kanterewicz BI, Hayes EL, and Klann E (2001) Peroxynitrite-induced tyrosine nitration and inhibition of protein kinase C. *Biochem Biophys Res Commun* **286**:764–770.
- Kuhn DM, Aretha CW, and Geddes TJ (1999) Peroxynitrite inactivation of tyrosine hydroxylase: mediation by sulfhydryl oxidation, not tyrosine nitration. *J Neuroscience* **19**:10289–10294.
- Kuhn DM and Geddes TJ (1999) Peroxynitrite inactivates tryptophan hydroxylase via sulfhydryl oxidation. *J Biol Chem* **274**:29726–29732.
- Kuhn DM, Sadidi M, Liu X, Kreipke C, Geddes T, Borges C and Watson JT (2002) Peroxynitrite-induced nitration of tyrosine hydroxylase: identification of tyrosines 423, 428 and 432 as sites of modification by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and tyrosine-scanning mutagenesis. *J Biol Chem* **277**:14336–14342.
- Lee C-I, Liu X, and Zweier J (2000) Regulation of xanthine oxidase by nitric oxide and peroxynitrite. *J Biol Chem* **275**:9369–9376.
- Lundqvist G and Morgenstern R (1992) Mechanism of activation of rat liver microsomal glutathione transferase by noradrenaline and xanthine oxidase. *Biochem Pharmacol* **43**:1725–1728.
- Lundqvist G, Yücel-Lindberg T, and Morgenstern R (1992) The oligomeric structure of rat liver microsomal glutathione transferase studied by chemical cross-linking. *Biochim Biophys Acta* **1159**:103–108.
- MacMillan-Crow LA, Crow JP, and Thompson JA (1998) Peroxynitrite-mediated inactivation of manganese superoxide dismutase involves nitration and oxidation of critical tyrosine residues. *Biochemistry* **37**:1613–1622.
- Morgenstern R and DePierre JW (1983) Microsomal glutathione transferase. Purification in unactivated form and further characterization of the activation process, substrate specificity and amino acid composition. *Eur J Biochem* **134**:591–597.
- Morgenstern R, DePierre JW, and Ernster L (1979) Activation of microsomal glutathione S-transferase activity by sulfhydryl reagents. *Biochem Biophys Res Commun* **87**:657–663.
- Morgenstern R, DePierre JW, and Ernster L (1980) Reversible activation of rat liver microsomal glutathione S-transferase activity by 5,5'-dithiobis(2-nitrobenzoic acid) and 2,2'-dipyridyl disulfide. *Acta Chem Scand* **34**:229–230.
- Morgenstern R, Guthenberg C, and DePierre JW (1982) Microsomal glutathione S-transferase. Purification, initial characterization and demonstration that it is not identical to the cytosolic glutathione S-transferase A, B and C. *Eur J Biochem* **128**:243–248.
- Morgenstern R, Lundqvist G, Andersson G, Balk L, and DePierre JW (1984) The distribution of microsomal glutathione transferase among different organelles, different organs and different organisms. *Biochem Pharmacol* **22**:3609–3614.
- Morgenstern R, Lundqvist G, Jörnval H, and DePierre JW (1989) Activation of rat liver microsomal glutathione transferase by limited proteolysis. *Biochem J* **260**:577–582.
- Mosialou E and Morgenstern R (1989) Activity of rat liver microsomal glutathione transferase toward products of lipid peroxidation and studies of the effect of inhibitors on glutathione-dependent protection against lipid peroxidation. *Arch Biochem Biophys* **275**:289–294.
- Otieno MA, Baggs RB, Hayes JD, and Anders MW (1997) Immunolocalization of microsomal glutathione S-transferase in rat tissues. *Drug Metab Dispos* **25**:12–20.
- Patel RP, McAndrew J, Sellak H, White CR, Jo H, Freeman BA and Darley-Usmar VM (1999) Biological aspects of reactive nitrogen species. *Biochim Biophys Acta* **1411**:385–400.
- Pryor WA and Squadrito GL (1995) The chemistry of peroxynitrite: a product from the reaction of nitric oxide with superoxide. *Am J Physiol* **268**:L699–L722.
- Redd CC, Tu C-PD, Burges JR, Ho C-Y, Sholz RW, and Massaro EJ (1981) Evidence for the occurrence of selenium-independent glutathione peroxidase activity in rat liver microsomes. *Biochem Biophys Res Commun* **101**:970–978.
- Riddles PW, Blakeley RL, and Zerner B (1983) Reassessment of Ellman's reagent. *Methods Enzymol* **91**:49–60.
- Savvides SN, Scheiwein M, Bohme CC, Arteel GE, Karplus PA, Becker K, and Schirmer RH (2002) Crystal structure of the antioxidant enzyme glutathione reductase inactivated by peroxynitrite. *J Biol Chem* **277**:2779–2784.
- Schwemmer M, Fink B, Kockbauer R, and Bassenge E (2000) How urine analysis reflects oxidative stress-nitrotyrosine as a potential marker. *Clin Chim Acta* **297**:207–216.
- Sies H, Därfre AL, Ji Y, and Akerboom TP (1998) Protein S-thiolation and redox regulation of membrane-bound glutathione transferase. *Chem Biol Interact* **111–112**:177–185.
- Sokolovsky M, Riordan JF, and Vallee BL (1966) Tetranitromethane. A reagent for the nitration of tyrosyl residues in proteins. *Biochemistry* **5**:3582–3589.
- Takakura K, Beckman JS, MacMillan-Crow LA, and Crow JP (1999) Rapid and irreversible inactivation of protein tyrosine phosphatases PTP1B, CD45 and LAR by peroxynitrite. *Arch Biochem Biophys* **369**:197–207.
- Viner RI, Willis TD, and Schöneich C (1999) Peroxynitrite modification of protein thiols: oxidation, nitration, nitrosylation and S-glutathiolation of functionally important cysteine residue(s) in the sarcoplasmic reticulum Ca-ATPase. *Biochemistry* **38**:12408–12415.
- Weinander R, Ekström L, Andersson C, Raza H, Bergman T, and Morgenstern R (1997) Structural and functional aspects of rat microsomal glutathione transferase. *J Biol Chem* **272**:8871–8877.
- Wong PS, Eiserich JP, Reddy S, Lopez CL, Cross CE and van der Vliet A (2001) Inactivation of glutathione S-transferases by nitric oxide-derived oxidants: exploring a role for tyrosine nitration. *Arch Biochem Biophys* **394**:216–228.
- Zou MH, Shi C, and Cohen RA (2002) Oxidation of the zinc-thiolate complex and uncoupling of endothelial nitric oxide synthase by peroxynitrite. *J Clin Invest* **109**:817–826.

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