Biotransformation of Glyceryl Trinitrate by Rat Hepatic Microsomal Glutathione S-Transferase 1

Yanbin Ji and Brian M. Bennett

Department of Pharmacology and Toxicology, Faculty of Health Sciences, Queen’s University, Kingston, Ontario, Canada

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ABSTRACT

Although the biotransformation of organic nitrates by the cytosolic glutathione S-transferases (GSTs) is well known, the relative contribution of the microsomal GST (MGST1) to nitrate biotransformation has not been described. We therefore compared the denitration of glyceryl trinitrate (GTN) by purified rat liver MGST1 and cytosolic GSTs. Although MGST1 and cytosolic GSTs catalyzed the denitration of GTN, the activity of MGST1 toward GTN was 2- to 3-fold higher. To mimic oxidative/nitrosative stress in vitro, we treated enzyme preparations with hydrogen peroxide, S-nitrosoglutathione, and peroxynitrite. Both oxidants and nitrating reagents increased the activity of MGST1 toward the GST substrate, 1-chloro-2,4-dinitrobenzene (CDNB) whereas these treatments inhibited GTN denitration by MGST1. Alkylation of the sole cysteine residue of MGST1 by N-ethylmaleimide markedly increased enzyme activity with CDNB as substrate but decreased the rate of GTN denitration. In aortic microsomes from GTN-tolerant animals, there was a decreased abundance of MGST1 dimers and trimers. In hepatic microsomes from GTN-tolerant animals, GTN biotransformation was unaltered whereas the rate of CDNB conjugation was doubled, suggesting that chronic GTN exposure causes structural modifications to the enzyme, resulting in increased activity to certain substrates. Collectively, these data indicate that MGST1 contributes significantly to the biotransformation of GTN and that chemical modification of the microsomal enzyme has differential effects on the catalytic activity toward different substrates.

Organic nitrates such as glyceryl trinitrate (GTN) have been used clinically for more than a century in the treatment of angina pectoris and congestive heart failure. It is generally accepted that GTN is a prodrug that requires bioactivation to form nitric oxide (NO), or a closely related species, before activation of guanylyl cyclase, increased cyclic GMP, and vascular smooth muscle relaxation. In addition to this mechanism-based biotransformation of GTN to activators of guanylyl cyclase, the denitration of GTN in vascular and nonvascular tissues to inorganic nitrite anion (NO2−) and its two dinitrate metabolites, glyceryl-1,2-dinitrate (1,2-GDN) and glyceryl-1,3-dinitrate (1,3-GDN) also occurs. This is catalyzed by a number of proteins and enzymes, including hemoglobin and myoglobin (Bennett et al., 1986), the cytochromes P450-NADPH cytochrome P450 reductase system (McDonald and Bennett, 1990, 1993; McGuire et al., 1998), xanthine oxidoreductase (Doel et al., 2000; Ratz et al., 2000b), old yellow enzyme (Meah et al., 2001), ALDH2 (Chen et al., 2002) and the glutathione S-transferases (GSTs) (Habig et al., 1975; Tsuchida et al., 1990; Nigam et al., 1996).

Although the GSTs are found primarily in the cytosol as homodimers or heterodimers, a membrane-bound GST (MGST1) that accounts for up to 3% of microsomal protein also has been identified (Morgenstern et al., 1982). The MGST1 is a member of the membrane-associated proteins in eicosanoid and glutathione metabolism superfamily of proteins, and although classified as a glutathione S-transferase, this GST isoform bears no obvious structural resemblance (amino acid sequence, molecular weight, or immunological properties) to the cytosolic GSTs.

Most previous studies have focused on the role of the cytosolic GSTs in the biotransformation of GTN (Habig et al., 1975; Posadas del Rio et al., 1988; Tsuchida et al., 1990; Kurz et al., 1993; Nigam et al., 1996). However, the GST-dependent biotransformation of GTN in hepatic microsomes also occurs (Lau and Benet, 1990), suggesting a role for MGST1 in GTN biotransformation. The interpretation of microsomal biotransformation studies is complicated by the association

ABBREVIATIONS: GTN, glyceryl trinitrate; NO, nitric oxide; GDN, glyceryl dinitrate; GST, glutathione S-transferase; GST1, microsomal glutathione S-transferase 1; GSH, reduced glutathione; CDNB, 1-chloro-2,4-dinitrobenzene; NEM, N-ethylmaleimide; GSNO, S-nitrosoglutathione; TBARS, thiobarbituric acid-like reactive substance(s); PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; ALDH2, aldehyde dehydrogenase-2.
of certain cytosolic GST isozymes with the microsomal fraction (Morgenstern et al., 1983; Hayes et al., 2005), and there is little information regarding the specific role of MGST1 in GTN biotransformation. A characteristic of rat liver MGST1 is that covalent modification (e.g., alkylation by N-ethylmaleimide) of its single cysteine residue (Cys-49) results in a marked increase in enzyme activity. Oxidative modification of Cys-49 also results in increased enzyme activity, and recent data from our laboratory have demonstrated that in addition to oxidative stress, nitrosative stress increases MGST1 activity by tyrosine nitration of Tyr-92 and, to a lesser extent, by nitrosylation of Cys-49 (Ji et al., 2002, 2006; Ji and Bennett, 2003). In the present study, our goal was to compare GTN biotransformation by the microsomal and cytosolic GSTs to assess the effect of various modifications of MGST1 on GTN biotransformation and to assess whether MGST1 activity or expression is altered in GTN-tolerant animals.

Materials and Methods

Materials. Rat liver cytosolic GSTs, 1-chloro-2,4-dinitrobenzene (CDNB), GSH, dithiothreitol, CM-Sepharose, hydroxyapatite, N-ethylmaleimide (NEM), and Triton X-100 were purchased from Sigma-Aldrich Canada Ltd (Oakville, ON, Canada). Manganese (IV) oxide was from Aldrich (Milwaukee, WI). TRIDIL GTN injection USP (5 mg/ml) in ethanol-propylene glycol-water (1:1:1.33) was obtained from Sabex Inc. (Boucherville, QC, Canada), and transdermal GTN patches were obtained as Transderm-Nitro brand (0.2 mg/h) from Abbott Laboratories (North Chicago, IL). Both Hybond-N membranes (Amersham, Arlington Heights, IL) and Hybond-C membrane (Amersham) were purchased from BioRad (Mississauga, ON, Canada). Polyclonal anti-rat MGST1 antiserum was raised in rabbits by Cocalico Biologicals Inc (Reamstown, PA) using purified rat hepatic MGST1 prepared in our laboratory. Chemiluminescence reagents were from Perkin-Elmer (Boston, MA). RNase Mini Kits were from QIAGEN Inc (Mississauga, ON, Canada), and QuikHyb Rapid Hybridization elution solution was from Stratagene (La Jolla, CA). Coomasie blue (Coomassie Brilliant Blue R-250, v/v) solution was from Sigma-Aldrich (St. Louis, MO). 1% agarose gels containing 1.1% formaldehyde was obtained from BioRad Laboratories (Richmond, CA). An 18% SDS-PAGE gel containing 1.1% formaldehyde was transferred to membranes at 30 V for 1 h. Phosphorimager gel imaging was performed with the Molecular Dynamics (Sunnyvale, CA) Storm 840.

Synthesis of GSNO and Peroxynitrite. GSNO was prepared by reacting equimolar concentrations of sodium nitrite and GSH as described previously (Ji et al., 1996). The concentration of GSNO was determined spectrophotometrically at 354 nm (ε = 767 M⁻¹ cm⁻¹) and stored at −70°C. Peroxynitrite was synthesized from acidified nitrite and H₂O₂ as described by Beckman et al. (1994) and stored at −70°C. The concentration of peroxynitrite was determined spectrophotometrically at 302 nm (ε = 1670 M⁻¹ cm⁻¹) at the time of synthesis and again just before use. The H₂O₂ contamination of peroxynitrite solutions was removed by manganese dioxide chromatography and filtration (Beckman et al., 1994).

Measurement of Microsomal Lipid Peroxidation. Lipid peroxidation products in hepatic microsomes from control and GTN-tolerant rats were determined spectrophotometrically at 532 nm (ε = 1.56 × 10⁵ M⁻¹ cm⁻¹) as thiobarbituric acid-like reactive substances (TBARS) (Aust, 1985).

Isolation of Rat Liver Microsomes and Purification of MGST1. Rat hepatic or aortic microsomes were prepared as described previously (McDonald and Bennett, 1993; Ji et al., 1996). Microsomes were washed twice with 100 mM Tris-HCl (pH 7.4) to decrease cytosolic contamination and then stored at −70°C. Hepatic MGST1 was purified by hydroxyapatite and CM-Sepharose chromatography by the method of Morgenstern and DePierre (1983) as described in Ji and Bennett (2003). GST activity was determined using the method of Habig et al. (1974). Samples (1.0 ml) contained 100 mM potassium phosphate, pH 6.5, 1 mM GSH, and 1 mM CDNB at 25°C. For the assay of MGST1 and microsomal fractions, 0.5% Triton X-100 was added to the incubation buffer.

GTN Biotransformation Studies. Chemical modification of cytosolic GSTs or MGST1 was performed by incubation of enzyme in 100 mM potassium phosphate (pH 7.0) with 1.0 mM NEM for 1 min or 2 mM peroxynitrite for 10 s at room temperature and with 2 mM GSNO for 10 min or 5 mM H₂O₂ for 30 min at 37°C (Aiyia and Anders, 1992; Ji et al., 2002; Ji and Bennett, 2003). To assess GTN biotransformation, 5 μg of purified cytosolic GSTs or MGST1 was incubated with the indicated concentrations of GTN and 1 mM GSH at 37°C for 60 min in a final volume of 0.5 ml. Some samples were pretreated with 1 mM NEM before assessment of GTN biotransformation. The GTN metabolites, 1,2-GDN and 1,3-GDN, were quantitated by gas chromatography with electron capture detection as described previously (McDonald and Bennett, 1990). Denitration rates were corrected for the nonenzymatic formation of GDNs (−5% of total metabolites).

SDS-PAGE and Immunoblot Analysis. Rat liver and aortic microsomes from control and GTN-tolerant animals were resolved on a 15% SDS-PAGE gel under nonreducing conditions. Proteins were transferred electrophoretically to PVDF membranes and then incubated with specific antiserum against rat liver MGST1 (1:5000). The membranes were then incubated with the indicated concentrations of GTN and 1 mM GSH at 37°C for 60 min in a final volume of 0.05 ml. Membranes were exposed to a phosphor storage phosphor screen at 68°C and then hybridized for 2 h at 68°C with cDNA probes ³²P-radiolabeled with ³²P-dCTP (6000 Ci/mmol) by random priming. The 599-base pair MGST1 cDNA probe was generated by reverse transcription-polymerase chain reaction procedure using rat liver as the source of RNA. Membranes were exposed to a phosphor storage screen at −70°C for 2 h, scanned with a Bio-Rad Molecular Imager FX (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada) and quantitated using Corel Photo-Paint version 8 software. After quantitation of MGST1 mRNA, membranes were stripped and reprobed with an 18 S rRNA probe to correct for variations in RNA loading.

Data Analysis. Data were analyzed by the appropriate statistical test as indicated. A p value of ≤0.05 was considered statistically significant.

Results

GTN Biotransformation Studies. Purified MGST1 and cytosolic GSTs both catalyzed the denitration of GTN to the
1,2- and 1,3-GDN metabolites (Fig. 1, A and B) and exhibited selectivity for the formation of 1,3-GDN. The specific activity of the MGST1 for GTN denitration was at least 2- to 3-fold greater than that of the rat liver cytosolic GSTs, with the greatest differences observed at low GTN concentration (1 μM). It is well documented that NEM markedly activates MGST1 but partially inhibits cytosolic GST activity when CDNB is used as the second substrate. In contrast, although NEM did inhibit GTN biotransformation by the cytosolic GSTs, it also inhibited GTN denitration by the microsomal enzyme (Fig. 1, A and B). The derived kinetic parameters for the GTN denitration reaction mediated by MGST1 were calculated using the data in Fig. 1 and gave values of 44 nmol/min/mg of protein for $V_{\text{max}}$ and 49 μM for $K_m$.

Effect of Oxidants and Nitrating Reagents on Microsomal and Cytosolic GST Activity. Structural modifications of the MGST1 result in increased activity toward the classic GST substrate, CDNB, and we wished to determine whether activity toward GTN was altered in a similar manner. As seen in Table 1, treatment of the MGST1 with a variety of modifying reagents resulted in an increase in activity toward CDNB, whereas when GTN was used as substrate, the denitration activity of the modified enzyme was decreased (with the exception of GSNO-treated enzyme). The most dramatic difference in activity occurred after alkylation of Cys-49 by NEM. In this case, activity toward CDNB was increased 12-fold whereas activity toward GTN was decreased by almost 90%. For the cytosolic GSTs, all of the protein-modifying reagents inhibited cytosolic GST activity toward both CDNB and GTN, albeit to differing degrees. Another striking difference between the two substrates was the greater specific activity of the cytosolic GSTs for CDNB relative to the MGST1, whereas for GTN the opposite was true.

Hepatic Microsomal and Cytosolic GST Activities in GTN-Tolerant Animals. To determine whether chronic exposure to GTN results in changes in hepatic GST activity, we assessed enzyme activity in subcellular fractions of the livers from naive and GTN-tolerant animals. For the cytosolic fraction, activity toward both CDNB and GTN was reduced by ~25% in preparations from GTN-tolerant animals. In contrast, GTN biotransformation was unaltered in microsomes from GTN-tolerant animals, and activity in control and tolerant microsomes was inhibited to the same degree after treatment of microsomes with NEM (Table 2). With CDNB as substrate, however, enzyme activity was increased ~2-fold in microsomes from GTN-tolerant animals, although the maximal activation after treatment of microsomes with NEM was the same for the two microsomal preparations. Because it has been suggested that oxidative/nitrosative stress occurs in GTN tolerance, one might predict that there would be increased lipid peroxidation as a consequence of chronic GTN exposure. We assessed the level of lipid peroxidation, as measured by TBARS levels, and found no difference between microsomes from naive and GTN-tolerant animals (0.20 ± 0.05 and 0.18 ± 0.04 nmol of TBARS/mg protein, respectively, $p > 0.05$, unpaired $t$ test, $n = 3$).

Immunoblot and Northern Analysis of MGST1. Immunoblot and Northern blot analyses were performed to determine whether chronic exposure to GTN resulted in changes in MGST1 expression. The data in Figs. 2 and 3 indicate that the hepatic MGST1 was down-regulated only at the mRNA level in GTN-tolerant animals. There was an ~60% reduction in mRNA levels, whereas the levels both the monomeric and dimeric forms of the enzyme were essentially unchanged. We also assessed MGST1 protein in aortic microsomes and found that there was a reduction in the dimeric form of the enzyme in microsomes from GTN-tolerant animals and also a reduction in the levels of MGST1 trimer. However, in contrast to the data from liver, there was an increase in the levels of MGST1 monomer in aortic microsomes from GTN-tolerant animals. The levels of aortic MGST1 mRNA from GTN-tolerant animals were somewhat variable, and although the average level of expression was doubled, this increase was not statistically significant.

Discussion

In the present study we wished to assess the relative rate of GTN biotransformation by MGST compared with the cytosolic GSTs and to determine whether MGST1 activity or expression is altered in GTN-tolerant animals. As seen in Fig. 1, the rate of denitration of GTN by MGST1 was significantly greater than that of the cytosolic GSTs, especially at lower, more pharmacologically relevant, concentrations of GTN. Thus, at an initial substrate concentration of 1 μM, the
TABLE 1
Comparison of rat liver microsomal and cytosolic GST activities towards CDNB and GTN

<table>
<thead>
<tr>
<th>Modifying Reagents</th>
<th>Modification</th>
<th>Enzyme Activity</th>
<th></th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>CDNB (μmol/min/mg protein)</td>
<td>GTN (×10⁻⁶)</td>
</tr>
<tr>
<td>Cytosolic GSTs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nontreated</td>
<td>NA</td>
<td>21 ± 3</td>
<td>3.4 ± 0.4</td>
</tr>
<tr>
<td>2 mM GSNO</td>
<td>S-Nitrosylation</td>
<td>10 ± 2</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>5 mM H₂O₂</td>
<td>S-Oxidation, S-glutathiolation</td>
<td>16 ± 3</td>
<td>0.61 ± 0.07</td>
</tr>
<tr>
<td>2 mM ONOO⁻</td>
<td>S-Oxidation, tyrosine nitrification</td>
<td>2.5 ± 0.7</td>
<td>0.51 ± 0.03</td>
</tr>
<tr>
<td>1 mM NEM</td>
<td>Alkylation</td>
<td>12 ± 1</td>
<td>0.82 ± 0.2</td>
</tr>
<tr>
<td>MGST1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nontreated</td>
<td>N.A.</td>
<td>1.0 ± 0.2</td>
<td>8.7 ± 0.6</td>
</tr>
<tr>
<td>2 mM GSNO</td>
<td>S-Nitrosylation</td>
<td>1.8 ± 0.1</td>
<td>8.9 ± 0.7</td>
</tr>
<tr>
<td>5 mM H₂O₂</td>
<td>S-Oxidation, S-glutathiolation</td>
<td>2.8 ± 0.4</td>
<td>2.8 ± 0.9</td>
</tr>
<tr>
<td>2 mM ONOO⁻</td>
<td>S-Oxidation, tyrosine nitrification</td>
<td>5.1 ± 0.5</td>
<td>3.9 ± 0.5</td>
</tr>
<tr>
<td>1 mM NEM</td>
<td>Alkylation</td>
<td>11 ± 2</td>
<td>1.0 ± 0.02</td>
</tr>
</tbody>
</table>

N.A., not applicable.

TABLE 2
Comparison of hepatic GST activities towards CDNB (1.0 mM) and GTN (1.0 μM) in cytosolic and microsomal fractions from three control and three GTN-tolerant rats

<table>
<thead>
<tr>
<th>Enzyme Activity</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>CDNB (μmol/min/mg protein)</td>
</tr>
<tr>
<td>Cytosol</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.82 ± 0.01</td>
</tr>
<tr>
<td>Tolerant</td>
<td>0.66 ± 0.02a</td>
</tr>
<tr>
<td>Micromesomes</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.043 ± 0.006</td>
</tr>
<tr>
<td>Control + NEM</td>
<td>0.174 ± 0.004b</td>
</tr>
<tr>
<td>Tolerant</td>
<td>0.091 ± 0.005b</td>
</tr>
<tr>
<td>Tolerant + NEM</td>
<td>0.171 ± 0.007b</td>
</tr>
</tbody>
</table>

a P < 0.05 versus control, unpaired Student’s t test.
b P < 0.01 versus control, one-way analysis of variance and Newman-Keuls post hoc test.

Fig. 2. Immunoblot analysis of hepatic MGST1 from control and GTN-tolerant rats. Purified MGST1 (10 ng) or hepatic microsomal protein (2 μg) from four control and four GTN-tolerant rats were resolved on a 15% SDS-PAGE gel under nonreducing conditions, transferred to PVDF membranes, and probed with a rabbit polyclonal antibody to rat MGST1.

rate of denitration by MGST1 was 3-fold greater than that by the cytosolic GSTs.

Both enzyme preparations exhibited regioselectivity for GTN denitration, with selective formation of the 1,3-GDN metabolite. Treatment of the cytosolic GSTs with the alkylating agent NEM resulted in a decrease in GTN denitration (Fig. 1, A and B) and a parallel decrease in the enzyme activity toward CDNB (Table 1). However, in marked contrast to the increase in MGST1 activity toward CDNB after alkylation of Cys-49 by NEM (Table 1), the GTN denitration activity of MGST1 activity toward GTN was inhibited markedly by NEM (Fig. 1, A and B).

Activation of MGST1 by covalent modification of Cys-49 is a characteristic that distinguishes the microsomal GST from its cytosolic counterparts (Morgenstern and DePierre, 1983). The MGST1 is thought to exist as a homotrimer in the native state. The trimer binds one molecule of GSH and altered communication between subunits is believed to play a role in enzyme activation by agents such as NEM. The formation of the thiolate consists of fast and slow components, with rapid communication between subunits is believed to play a role in enzyme activation by agents such as NEM. The formation of the thiolate consists of fast and slow components, with rapid binding of GSH followed by slower formation of thiolate (Svensson et al., 2004). Modification of Cys-49 by NEM increases the rate of thiolate formation and increases kcat. The activation of MGST1 by NEM with respect to CDNB conjugation is in marked contrast to the inhibition of GTN bio-transformation after alkylation of the enzyme with NEM and suggests a differential interaction of the two substrates with the enzyme. In previous studies examining the kinetic properties of the NEM-activated enzyme, it was found that an increase in the rate of catalysis occurred only with certain substrates and that with others it was unaltered and that...
this was related to the relative reactivity of substrate (Morgenstern and DePierre, 1983; Morgenstern et al., 1988). Although GTN is a substrate for the enzyme, the specific activity for denitrification is quite low compared with the rate of the conjugation reaction with CDNB. However, this lower specific activity would not explain the observed decrease in enzyme activity after treatment with NEM.

Another possible mechanism for the inhibitory effect of NEM is a direct interaction of the organic nitrate with the free SH groups of the enzyme, resulting in denitration, and prevention of this interaction by alkylation of the sulfhydryl group by NEM. We examined whether other modifications of the enzyme resulted in changes in activity toward GTN and, as seen in Table 1, treatment of the MGST1 with reagents that would alter Cys-49 also inhibited GTN denitrification activity. Thus, a direct interaction of GTN with Cys-49 seems possible.

Tolerance to GTN after chronic exposure in vivo is associated with attenuation of the vasodilator responses to GTN and also decreases in the vascular biotransformation of GTN (Ratz et al., 2000a). In addition, the pattern of GTN metabolite formation in the whole animal is altered in GTN-tolerant animals (Ratz et al., 2002). To assess whether chronic GTN exposure might alter the contribution of MGST1 to the overall biotransformation of GTN, we compared the activity and expression of MGST1 in hepatic microsomes from naive and GTN-tolerant animals. The levels of MGST1 mRNA were significantly reduced in the livers of GTN-tolerant animals, although the level of expression of MGST1 protein was unchanged (Figs. 2 and 3). However, the levels of MGST1 protein did not correlate well with enzyme activity toward CDNB. With respect to GTN, there was no change in the rate of metabolite formation whereas for CDNB conjugation, GST activity was actually increased despite the fact that MGST1 protein expression was unchanged. This finding suggests that the chronic exposure to GTN resulted in some sort of structural or conformational change in the enzyme that resulted in an increase in catalytic activity toward CDNB but not to GTN. An alternative explanation could be that the expression of microsome-associated cytosolic GSTs is increased during chronic GTN exposure and that this increase accounts for the increase in GST activity. It is noteworthy that the expression of the class Alpha GST present in human hepatic microsomes is increased after exposure of cultured human hepatoblastoma (HepG2) cells to the prooxidant, hydrogen peroxide (Prabhu et al., 2004). With respect to the cytosolic GSTs, biotransformation of GTN was decreased in

the hepatic cytosolic fraction from GTN-tolerant animals, as was the enzyme activity of the cytosol toward CDNB. A similar degree of inhibition of CDNB conjugation was found after incubation of purified enzyme preparations with GTN (Lee and Fung, 2003).

As was observed with the purified MGST1, treatment of hepatic microsomes with NEM markedly inhibited GTN denitrification, and this occurred to a similar extent in microsomes from control and GTN-tolerant animals. In contrast, in a previous study it was found that incubation of hepatic microsomes with the alkyl nitrites, amyl nitrite and n-butyl nitrite, resulted in the formation of GSNO, and that treatment of microsomes with NEM increased the rate of GSNO formation (Ji et al., 1996). These data suggest that subtle changes in substrate structure have profound effects on catalysis by MGST1 and that the interaction of organic nitrates and organic nitrates with MGST1 is fundamentally different.

It was not technically feasible to generate sufficient quantities of aortic microsomes to assess changes in MGST activity. However, we were able to assess changes in aortic MGST1 expression after chronic GTN exposure. In contrast to the findings in liver, mRNA levels were unchanged in aortae from GTN-tolerant animals and levels of the MGST1 monomer were increased rather than unchanged (Figs. 4 and 5). In situ, MGST1 is thought to exist as a homotrimer (Lundqvist et al., 1992), although on both reducing and non-reducing SDS-PAGE gels the enzyme migrates primarily as the 17-kDa monomer. As seen in the immunoblots of hepatic and aortic microsomes from naive animals, immunoreactive

**Fig. 4.** Immunoblot analysis of aortic MGST1 from control and GTN-tolerant rats. Purified MGST1 (0.12 µg) or aortic microsomal protein (30 µg) from control and four GTN-tolerant rats were resolved on a 15% SDS-PAGE gel under nonreducing conditions, transferred to PVDF membranes, and probed with a rabbit polyclonal antibody to rat MGST1.

**Fig. 5.** Northern blot analysis of rat aortic MGST1. Northern analysis of rat aortic RNA (20 µg of total RNA per lane) from four control and three GTN-tolerant animals. The signal ratio of MGST1/18S rRNA was approximately 2-fold higher in aortae from GTN-tolerant animals, but this was not significantly different from control.
bands are evident at mobilities consistent with MGST1 dimers, and in the aorta as trimers as well, suggesting that a proportion of monomers are covalently linked to one another. However, in aortic microsomes from GTN-tolerant animals, there was a clear reduction in the dimeric and trimeric forms of the enzyme as seen on SDS-PAGE gels. The functional significance of this finding is uncertain, but the observation that the relative proportions of the enzyme in dimeric or trimeric forms is altered suggests that chronic exposure to GTN changes the association properties of the MGST1 monomer.

Using the data in Table 1, we derived a \( k_{cat}/K_m \) value for GTN of 754 M\(^{-1}\)S\(^{-1}\) for MGST1 and 263 M\(^{-1}\)S\(^{-1}\) for the cytosolic GST mixture, approximately a 3-fold difference. In comparison, the \( k_{cat}/K_m \) values for rat hepatic cytochromes P450-mediated GTN biotransformation in hepatic microsomes under aerobic and anaerobic conditions are 390 and 14,000 M\(^{-1}\) S\(^{-1}\), respectively (McDonald and Bennett, 1990), and that for purified recombinant human aldehyde dehydrogenase-2 (Li et al., 2006) is 1390 M\(^{-1}\) S\(^{-1}\). Thus, the catalytic efficiencies of four enzymes known to biotransform GTN are within a 5-fold range of each other (with the exception of cytochromes P450 under anaerobic conditions).

With respect to the overall quantitative contribution of these enzymes to GTN biotransformation, this also would be significantly influenced by the relative abundance of different biotransformation enzymes in a particular tissue. Microsomal protein accounts for ~20% of the protein in rat hepatocytes (DePierre and Dallner, 1975) and MGST1 comprises ~3% of microsomal protein (Morgenstern et al., 1982). On the other hand, the cytosolic GSTs make up ~5% of the cytosolic protein, and, thus, if one takes into account the higher activity but lower abundance of the MGST1 versus the lower activity and higher abundance of the cytosolic GSTs, the relative contributions would be within a 2-fold range of each other. The cytochromes P450 content of microsomes is approximately double that of MGST1 and the \( k_{cat}/K_m \) value (under aerobic conditions) is approximately half, and therefore their relative contribution would be approximately the same as that of MGST1. However, because GTN biotransformation by cytochromes P450 is markedly greater under anaerobic conditions, the relative contribution of cytochromes P450 would be expected to be sensitive to changes in the intracellular \( O_2 \) concentration. Mitochondrial protein accounts for ~7% of rat hepatocyte protein (Colbeau et al., 1971), and although ALDH2 has a relatively high \( k_{cat}/K_m \) value, its abundance would be low because of its mitochondrial location. There are other factors that complicate extrapolation of enzyme reaction rates to the in vivo situation. The lipophilic nature of GTN would predict its preferential subcellular distribution to membranes and thus better access to membrane-bound biotransformation enzymes, and, in addition, the rate of GTN biotransformation is markedly reduced in broken cell preparations compared with that in intact cells (Bennett et al., 1994). With respect to the functional significance of MGST1-mediated GTN biotransformation, we have found that in a stably transfected cell line that overexpresses rat MGST1, there was an increase in GTN-induced cGMP formation compared with wild-type cells, suggesting that the MGST1 can mediate the mechanism-based biotransformation of GTN to an activator of guanylyl cyclase (Y. Ji and B. M. Bennett, unpublished observations).

In summary, we have demonstrated that MGST1 can catalyze the denitration of GTN and that it does so with greater efficiency than its cytosolic counterparts. Chemical modification of MGST1 has differential effects on the catalytic activity toward different substrates, and chronic GTN exposure seems to cause structural modifications to the enzyme that, although having no effect on the biotransformation of GTN, result in increased activity toward other MGST1 substrates.

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References


Address correspondence to: Dr. Brian M. Bennett, Department of Pharmacology and Toxicology, Faculty of Health Sciences, Queen’s University, Kingston, Ontario, Canada K7L 3N6. E-mail: brian.bennett@queensu.ca