Glutathione *S*-Transferase-Mediated Metabolism of Glyceryl Trinitrate in Subcellular Fractions of Bovine Coronary Arteries

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The possible role of glutathione *S*-transferases (GTSs) in vascular glyceryl trinitrate (GTN) metabolism was investigated. GTN degradation to form its dinitrate metabolites (GDNs) in the 9000*g* (9k) supernatant fraction of bovine coronary arteries (BCA) was examined. BCAs were homogenized with a 3× volume of phosphate buffer, and the 9k fraction was obtained by centrifugation. GTN (40 ng/ml; 1.76×10^{-7} *M*) was incubated for 2 hr in the 9k fraction of BCA in the presence of reduced glutathione (2×10^{-3} *M*). Samples were taken at 10, 20, 40, 60, and 120 min. GTN was observed to degrade readily, exhibiting a half-life of 26 min in the incubate. While both 1,2- and 1,3-GDNs were generated from GTN, formation of 1,3-GDN was predominant (GDN ratio, as 1,2/1,3-GDN, = 0.7–0.8). Coincubation with 2×10^{-5} *M* concentrations of two GST inhibitors, sulfobromophthalein (SBP) and ethacrynic acid (ECA), decreased the rate of GTN loss. The GTN half-lives in SBP- and ECA-treated incubations were 66 and 84 min, respectively. In addition, the pattern of GDN formation was also altered. The resultant GDN ratios exceeded unity in the presence of these inhibitors, indicating that 1,3-GDN formation was attenuated to a greater extent than that of 1,2-GDN. These data suggest that vascular GTN metabolism in BCA is carried out by cytosolic GST isozymes which possess a preference for C-2 denitration of GTN.

KEY WORDS: glyceryl trinitrate; nitroglycerin; glutathione *S*-transferase; sulfobromophthalein; ethacrynic acid.

INTRODUCTION

Glyceryl trinitrate (GTN), or nitroglycerin, is a commonly prescribed organic nitrate vasodilator for the treatment of angina pectoris. The metabolism of GTN and other organic nitrates by vascular tissues is believed to be an important step in eliciting the vasodilatory effects of the drug (1). It has been hypothesized that denitration of GTN leads to the formation of nitrite ions, which subsequently undergo further biotransformation to generate vasoactive intermediates such as *S*-nitrosothiols (2) and nitric oxide (3,4). The crucial enzyme(s) responsible for the metabolism of GTN in vascular tissues, however, has not been identified. It has been postulated that glutathione *S*-transferase (GST) can metabolize GTN (5), and the mechanism for an interaction between glutathione (GSH) and GTN has been proposed (6). Previous studies from our laboratory have also suggested that various GST isozymes can be involved in the hepatic metabolism of GTN (7,8). GST has recently been reported to be present in vascular tissues (9). Therefore, it is possible that GST represents an important enzymatic pathway for organic nitrate metabolism and may be a decisive component of the mechanism of action for these agents in blood vessels. In this study, disrupted tissue preparations of bovine coronary artery (BCA) were used to investigate the vascular metabolism of GTN. The possible role of GST was examined by observing the effects of two GST substrate inhibitors, sulfobromophthalein (SBP) and ethacrynic acid (ECA), on GTN metabolism in BCA preparations.

MATERIALS AND METHODS

GTN solutions were prepared from Tridil solutions (America Critical Care, McGaw Park, IL), used commonly for intravenous GTN administration (5 mg/ml). The 1,2- and 1,3-glyceryl dinitrate metabolites (GDNs) were generously supplied by Marion Laboratories (Kansas City, MO). Potassium phosphate (monobasic) and sodium phosphate (dibasic) were obtained from Fisher (Fairlawn, NJ). GSH, SBP, and ECA were all purchased from Sigma (St. Louis, MO). The organic solvents used in the extraction procedure, i.e., pentane, methyl-*t*-butyl ether, and butyl acetate, were purchased from EM Sciences (Cherry Hill, NJ). All chemicals were purchased at the highest grade of purity obtainable.

Fresh bovine hearts were obtained from Ferrara Meat Market (San Jose, CA). The interventricular and the circumflex coronary arteries were dissected and placed in ice-cold phosphate buffer (0.13 *M* KH_2PO_4–Na_2HPO_4). Blood vessels from six bovine hearts were pooled together to provide the tissues needed for one study. The blood vessels were then sliced, chopped, and homogenized in 3 vol of phosphate buffer by a blade homogenizer (Brinkman Instruments, Westbury, NY). The blood vessel homogenates were centrifuged at 9000*g* and the supernatant layer was decanted and saved for further ultracentrifugation at 105,000*g* at 4°C. The 105,000*g* supernatant (cytosolic fraction) and pellet (microsomal fraction) were separated and recentrifuged two more times to minimize contamination between the two subcellular fractions. The various BCA fractions were stored at −70°C prior to the incubation experiments.

In each incubation, 5 ml of BCA 9000*g* supernatant was used. In the studies intended for the comparison of GTN metabolism between the cytosolic and the microsomal fractions, the protein concentration in the incubates was normalized to 4.0 mg/ml, using the protein assay of Lowry *et al.* (10). The incubates were placed in a water bath with the temperature maintained at 37°C. GSH (2 m*M*) was added as the cofactor for the metabolic reaction. The mixture was then preincubated for 5 min. For the inhibitor studies, 2×10^{-5} *M* SBP or ECA was added just before the 5-min preincubation period. Following preincubation, 40 ng/ml (1.76×10^{-7} *M*) GTN was added to the incubates. Samples (500 μl) were taken at 10, 20, 40, 60, and 120 min and were frozen immediately in a mixture of dry ice and methanol.

The concentrations of GTN and GDNs were determined as described previously (7). Briefly, samples were extracted three times with 10-ml mixtures of pentane and methyl-*t*-butyl ether (8:2). The organic phase was almost evaporated

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to dryness and 50 μl of *n*-butyl acetate was used for reconstitution of samples. A 0.3-μl aliquot was injected onto Varian 6000 and 6500 gas chromatographs (GCs) equipped with electron capture detectors. A standard curve of 0.5–20 ng was constructed, with *o*-iodobenzyl alcohol as the internal standard. Linearity was observed over this range, and both GTN and GDNs were clearly separated.

Due to the presence of nonenzymatic GTN degradation in phosphate buffer, a vial containing the phosphate buffer and 2 m*M* GSH was incubated with GTN in each study as a control. The extent of nonenzymatic degradation at each sampling time was subtracted from the observed metabolism in the experimental samples to account for metabolites resulting from nonenzymatic GTN degradation.

RESULTS

Figure 1a depicts the profile of GTN degradation and GDN formation in incubations with the 9000*g* supernatant fraction of BCA. GTN was metabolized via an apparent first-order degradation process ($t_{1/2}$ = 25.7 min). Concentrations of the GDN metabolites were found to increase as GTN was degraded, with 1,3-GDN being the predominant metabolite. When the amounts of GDNs resulting from nonenzymatic degradation were subtracted from these results, as shown in Fig. 1b, 1,3-GDN was still the predominant product. The mean GDN ratio (1,2-GDN/1,3-GDN) was found to be between 0.7 and 0.8. In the absence of 2.0 m*M* GSH, the rate of GTN metabolism was significantly lower (data not shown), suggesting that GTN was degraded via a thiol-dependent pathway.

The cytosolic fraction and the microsomal fraction of BCA were compared for their capacity in metabolizing GTN at an equimolar protein concentration. Figure 2a depicts the profiles of GTN degradation in buffer, microsomes, and cytosol. The cytosolic fraction exhibited a significantly higher rate of GTN degradation than microsomes. The amounts of GDNs, expressed as percentages of the initial GTN dose, at the end of the 120-min incubation, are listed in Table I. The

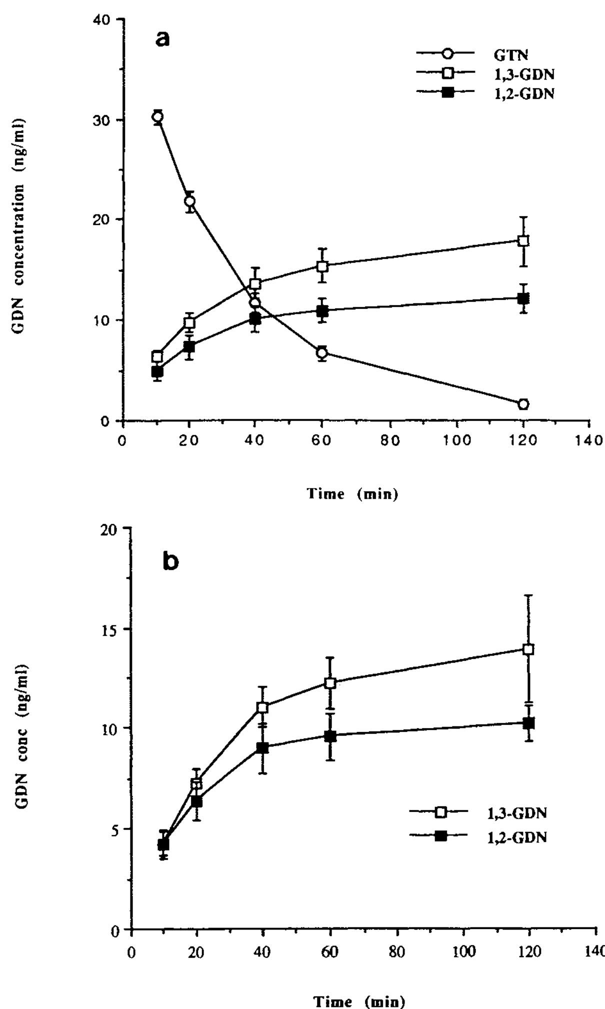

Fig. 1. Metabolism of GTN in the 9000*g* supernatant fraction of bovine coronary arteries. (a) Degradation of GTN and formation of 1,2- and 1,3-GDNs from a 40 ng/ml GTN incubation. (b) Formation of the dinitrate metabolites, after correction for the nonenzymatic component. Mean ± SD; *n* = 3.

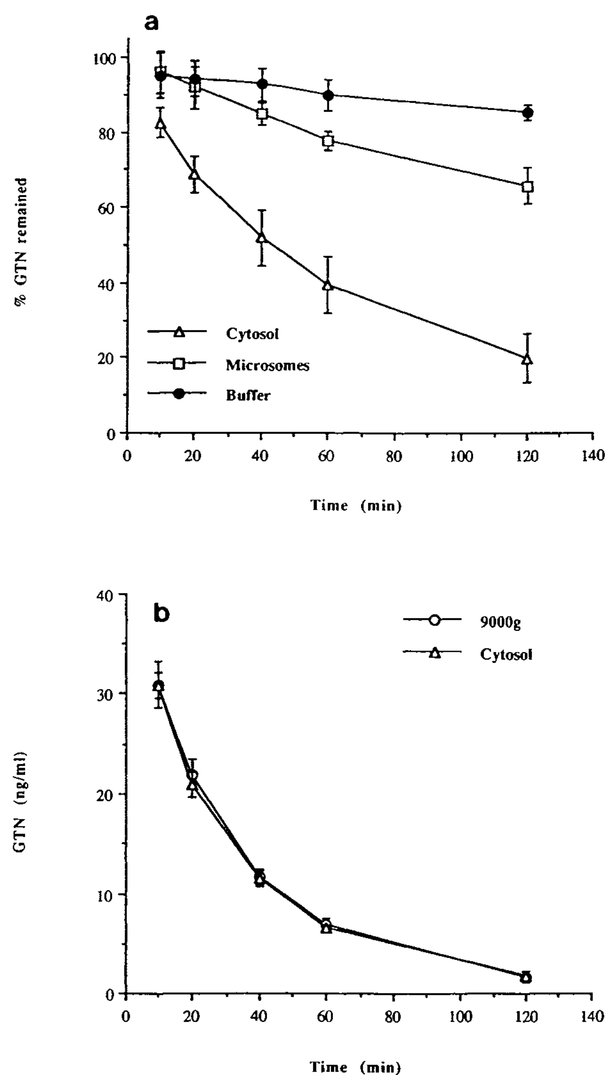

Fig. 2. (a) Metabolism of GTN in the cytosolic and the microsomal fractions of bovine coronary arteries (each at 4.0 mg/ml protein), compared to the buffer control. (b) Comparison of GTN degradation in 5 ml of 9000*g* and 105,000*g* supernatants, without normalization to protein content. Mean ± SD; *n* = 3.

Table I. Formation of GDN Metabolites from 40 ng/ml GTN Incubations in the Cytosolic and the Microsomal Fractions at Equimolar Protein Concentrations (Corrected for GDN Formation from Nonenzymatic GTN Degradation)[a]

	105,000*g* supernatant (cytosol)	105,000*g* pellet (microsomes)
% total GDN formed	65.3 ± 8.4	19.7 ± 6.5
% 1,2-GDN formed	26.7 ± 4.5	8.2 ± 3.5
% 1,3-GDN formed	38.6 ± 4.4	11.5 ± 3.4
% total protein in 9000*g* supernatant	70.1 ± 6.5	7.0 ± 0.9

[a] The percentage of total protein accounted for in each fraction, as a percentage of the total protein content in the 9000*g* supernatant is also shown (mean ± SD; *n* = 3).

extent of GTN degradation, as accounted for by the total metabolite formation, was three times higher in the cytosol than the microsomes. However, both fractions yielded 1,3-GDN as the predominant metabolite. The protein content in each subcellular fraction was determined and is expressed as a percentage of the total protein amount in the 9000*g* supernatant. The cytosol constituted the majority of the protein content in the 9000*g* supernatant, approximately 10-fold greater protein content than the microsomes. Thus considering the much higher rate of GTN metabolism observed in the cytosolic fraction and the predominance of cytosolic protein, these results suggest that the principle site of GTN metabolism in BCA is in the cytosol. The cytosolic fraction of BCA was compared to the 9000*g* fraction for the rate of GTN metabolism without normalizing to protein content (Fig. 2b). No difference was observed between the GTN degradation profiles of the two fractions, further indicating that the cytosol is responsible for the majority of GTN metabolism in BCA.

The 9000*g* supernatant fraction was incubated with GTN in the presence (2×10^{-5} *M*) of two GST substrate inhibitors—SBP and ECA. Figure 3 demonstrates the effects of these inhibitors on the rate of GTN degradation. The rate of GTN elimination was attenuated in the presence of both SBP and ECA. The first-order half-life of GTN elimination increased from 25.7 min in the control to 66.0 and 84.3 min for SBP- and ECA-treated incubates, respectively. The pattern of GDN formation was also altered in the presence of these inhibitors. Figures 4 a and b depict the effects of SBP and ECA on the formation of 1,2-GDN and 1,3-GDN, respectively. The concentrations of both GDNs were decreased compared to the control; however, the decrease in 1,3-GDN formation was more extensive than that of 1,2-GDN formation. As a result, the GDN ratio (1,2-GDN/1,3-GDN) increased in the presence of these GST inhibitors.

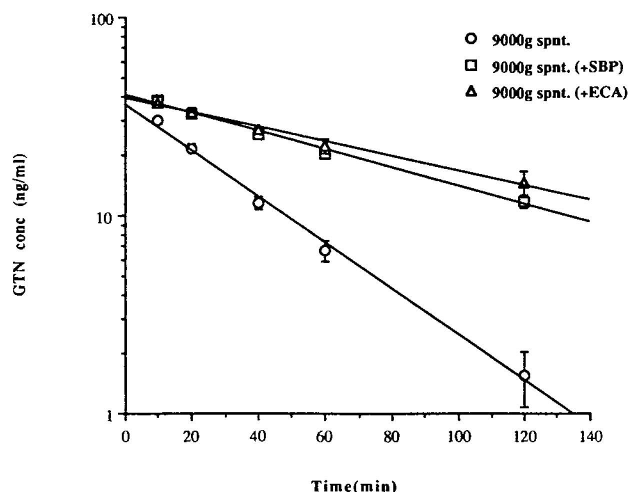

Fig. 3. Effects of sulfobromophthalein (SBP) and ethacrynic acid (ECA) on GTN degradation (40 ng/ml) in the 9000*g* supernatant (9000*g* spnt.) fractions of bovine coronary arteries. Mean ± SD; *n* = 3.

DISCUSSION

According to the *S*-nitrosothiol hypothesis (1), GTN and other organic nitrates must undergo denitration for the subsequent generation of the pharmacologically active species

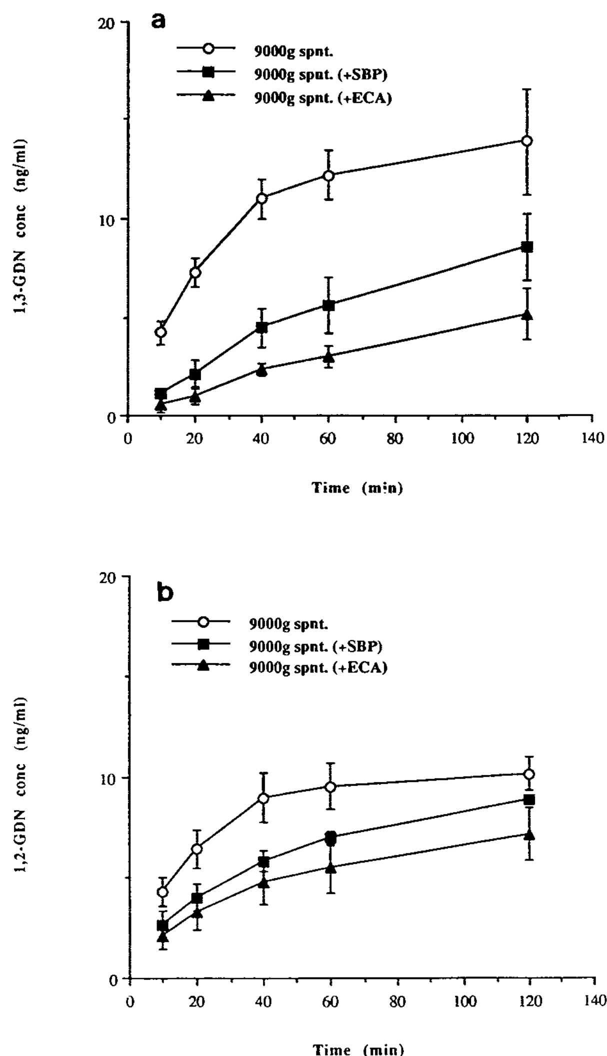

Fig. 4. Formation of (a) 1,3-GDN and (b) 1,2-GDN in the 9000*g* supernatant fraction of bovine coronary arteries from incubations with 40 ng/ml GTN in the presence or absence of sulfobromophthalein (SBP) and ethacrynic acid (ECA). The values of nonenzymatic GDN formation were subtracted (mean ± SD; *n* = 3).

(nitric oxide or *S*-nitrosothiol), which lead to the generation of the vasodilatory effects. Although the intermediary steps in the cascade of reactions have not been universally agreed upon, the relationship between the vascular metabolism of these agents and the generation of the vasorelaxing effects has been reported by various investigators (11–14). The identity of the enzyme(s) responsible for this denitration process has not been clarified. In this study, GTN metabolism in subcellular fractions of bovine coronary arteries was examined, and the possibility of the involvement of GST in vascular GTN metabolism was explored.

Coronary arteries from bovine hearts were used here because of the relatively higher yield in the amount of vascular tissues, thereby allowing various incubation studies with different subcellular fractions and different inhibitors of GTN metabolism. 1,3-GDN was found to be the slightly preferred metabolite from GTN denitration, indicating a regiopreference for C-2 denitration on the GTN molecule in these tissues. In addition, the cytosolic fraction was found to represent the major site of GTN metabolism. Upon the addition of two GST substrate inhibitors—SBP and ECA—GTN metabolism was attenuated, and the pattern of GDN formation was also altered. These results suggest the involvement of certain GST isozymes, which can be inhibited by SBP and ECA, in the vascular metabolism of GTN and possibly other organic nitrates.

In a previous study of GTN metabolism by intact bovine pulmonary veins, Marks *et al.* (15) reported preferential formation of 1,2-GDN. However, the regioselectivity was lost upon homogenization of the tissue (15). Thus, the metabolic scheme of GTN may vary depending on the preparation; the possibility that certain enzymatic pathways are activated or inactivated during homogenization cannot be ruled out. However, the biochemical characterization of the enzyme systems involved in GTN metabolism requires the elucidation of the metabolic pathways of GTN in homogenized tissue preparations. The C-2 denitrating regioselectivity reported here was similarly observed in homogenized blood vessel preparations from sheep (16) and rats (17).

In the studies described here, the concentration of the inhibitors used in the incubations was 100-fold higher than that of GTN (the substrate) and 100-fold less than the concentration of GSH (the cofactor). Therefore, the inhibition of GTN metabolism, as observed in the presence of these agents, should not be due to a depletion of the cofactors. In addition, the inhibitors did not change the nonenzymatic component of GTN degradation in buffer. Both SBP and ECA decreased the rate of GTN metabolism and preferentially inhibited the formation of 1,3-GDN, indicating that these agents blocked an enzymatic pathway of GTN metabolism mediated by GST. The results here in BCA differ from previous studies from our laboratory in rabbit liver cytosol, where SBP and ECA were found to inhibit 1,2-GDN formation more prominently, and where 1,2-GDN was the predominant metabolite from GTN metabolism (7,8). The discrepancy could be explained by possible interspecies and/or intertissue differences in the GST enzyme, hence leading to different regioselectivities on GTN denitration. GST has been known to exist as various isozyme forms in different organs (18) and different species (19).

Compared to hepatic GTN metabolism (7,8), the microsomal fraction contributed only in a minor way to the metabolism of the drug in BCA. The rate and extent of GTN metabolism were significantly lower in microsomes when equivalent amounts of protein were used in the incubations (Table I). Moreover, the yield of microsomal protein from the 9000*g* supernatant fraction was approximately 10 times lower than that of cytosolic protein. These data suggest that the glutathione-dependent pathway of GTN metabolism in vascular smooth muscles is in the cytosol. Chung and Fung (20), as well as Bennett *et al.* (21), have suggested that membrane-bound fractions may contain the essential enzymes responsible for GTN metabolism and activation. However, other investigators have demonstrated that activation of guanylate cyclase can occur solely in the presence of the cytosolic fractions from blood vessels (2,22), suggesting that the "productive" GTN metabolism is likely to occur within the cytoplasm of vascular smooth muscle cells. Although it cannot be assumed with certainty that the cytosolic GST-mediated metabolizing pathway observed here also represents the crucial pharmacological activation process, results from this study warrant further examination of the role of GSTs in mediating vascular GTN metabolism, as well as their relationship to the production of pharmacological effects. Future studies will examine the effects of GST inhibitors on *in vitro* measures of the pharmacodynamic responses to GTN, such as vasorelaxation and cGMP elevation, in animal blood vessel preparations.

Recently, GSTs, particularly the mu isozymes, were reported to be related to GTN metabolism (23,24). SBP has historically been considered to be predominantly a GST-mu substrate (25,26). ECA, although previously identified as a GST-pi substrate (26), has been found recently to be more effective as an inhibitor of class mu isozymes (27,28). Although the cross-reactivities of these inhibitors do not allow a definite identification of the class of GST isozymes involved in GTN metabolism in blood vessels, the inhibition of GTN metabolism by these agents, as observed here, demonstrates the inhibition of GST isozymes which are responsible for GTN metabolism. Recent evidence from our laboratory suggests that GST-mediated vascular GTN metabolism may also represent the activation pathway responsible for the generation of the pharmacologically active species which is necessary for GTN-induced vasorelaxation (29).

In summary, GTN was found to be metabolized by disrupted tissue preparations of BCA, with 1,3-GDN as the predominant metabolite. The majority of GTN metabolism in BCA was carried out in the cytosolic fraction, which yields a similar ratio of GDN metabolites as found in the 9000*g* supernatant. Upon the addition of SBP and ECA, the rate of GTN metabolism was drastically reduced, and the GDN ratio was altered, suggesting the involvement of GSTs in the vascular metabolism of GTN.

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