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METABOLISM OF NITROGLYCERIN BY SMOOTH MUSCLE CELLS

INVOLVEMENT OF GLUTATHIONE AND GLUTATHIONE *S*-TRANSFERASE

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Abstract—Metabolism of nitroglycerin (GTN) in the vascular smooth muscle is required for the drug to be effective in the treatment of angina pectoris and congestive heart failure. The usefulness of GTN is limited by the development of tolerance to the drug. The metabolism of GTN was studied in its target tissue, vascular smooth muscle. Inorganic nitrite was produced by cultured smooth muscle cells when GTN was added to the culture dish. Nitrite production increased with increasing GTN concentration and with incubation time. The enzymatic nature of GTN metabolism to nitrite was assessed by enzyme inhibition studies. Indocyanine green, a non-substrate inhibitor of glutathione *S*-transferase, inhibited GTN metabolism by smooth muscle cells. Cellular glutathione is also involved in GTN metabolism by the smooth muscle cell. Pretreatment with phorone, a glutathione *S*-transferase substrate, depleted cellular glutathione and decreased nitrite production from GTN. Pretreatment with buthionine sulfoximine, an inhibitor of γ-glutamylcysteine synthetase, decreased intracellular glutathione and caused decreased GTN metabolism in smooth muscle cells. Removal of cysteine from the smooth muscle cell incubation medium in combination with buthionine sulfoximine pretreatment decreased GTN metabolism to a lower level than buthionine sulfoximine pretreatment alone. This study shows that glutathione *S*-transferase and glutathione are involved in GTN metabolism by cultured smooth muscle cells.

Nitroglycerin (GTN‖) is used to treat angina pectoris and congestive heart failure. Its continuous use is limited by the development of tolerance to the drug in which its vasodilating effects are lost. Tolerance is considered to be related to GTN metabolism because a drug-free period of several hours restores efficacy. Elucidation of the mechanism by which tolerance develops should lead to rational approaches to its prevention. Several groups have proposed schemes for the metabolism of GTN [1–3]. Most studies have focused on steps subsequent to the reactions that produce nitrite. We have examined nitrite production.

The cellular mechanisms by which nitrite and subsequently nitric oxide are produced from GTN have not been elucidated completely; however, *in vitro* studies have demonstrated that GTN is a substrate of the glutathione *S*-transferases [4]. A scheme for GTN metabolism which includes this enzymatic step is shown in Fig. 1. Glutathione is required as a substrate for glutathione *S*-transferase activity (Fig. 1, step 1) and it is also proposed to be involved in the non-enzymatic production of nitrite from the intermediate, glutathione sulfenyl nitrite ($GS\text{-}NO_2$) (Fig. 1, step 2). Several reports have presented evidence showing the involvement of glutathione *S*-transferase in the initial metabolic activation of GTN. In studies with purified glutathione *S*-transferase, Jakoby and coworkers demonstrated that several of the glutathione *S*-transferase isoforms catalyze the conversion of GTN to nitrite and glyceryl dinitrate (GDN) with the simultaneous conversion of reduced glutathione to oxidized glutathione (GSSG) [4, 5]. They proposed the formation of $GS\text{-}NO_2$ as an intermediate in the overall reaction. Experiments by Lau and Benet [6] with subcellular fractions from rabbit liver suggested that different glutathione *S*-transferase isoforms in the cytosol and in the microsomal fraction are responsible for the metabolism of GTN to GDN in the two subcellular compartments. Yeates *et al.* [7] have reported that inhibition of glutathione *S*-transferase activity by bromosulfophthalein results in decreased GTN-induced relaxation of precontracted rabbit thoracic aorta strips. Sato and coworkers have isolated and characterized several distinct isoforms of glutathione *S*-transferase from human heart and aorta that can metabolize GTN [8].

The requirement for glutathione in GTN metabolism was studied by Needleman and Harkey [9] in

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‖ Abbreviations; GTN, nitroglycerin; GDN, glyceryl dinitrate; $GS\text{-}NO_2$, glutathione sulfenyl nitrite; GSSG, oxidized glutathione; SMC, smooth muscle cells; HBSS, Hanks' balanced salt solution; PBS, phosphate-buffered saline; ICG, indocyanine green; BSO, buthionine sulfoximine; and DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

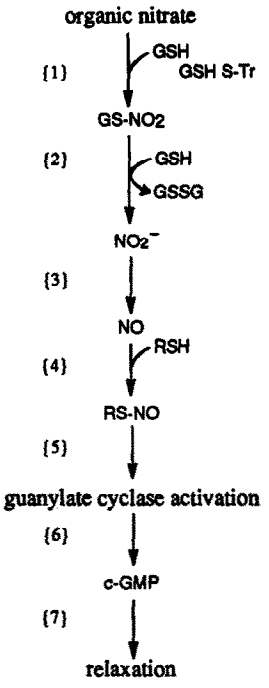

Fig. 1. Proposed mechanism for GTN metabolism in SMC. The first step is the enzymatic metabolism of GTN by glutathione *S*-transferase to produce GS-NO_2 {1}. This is followed by the non-enzymatic cleavage of GS-NO_2 by reaction with reduced glutathione (GSH) to give GSSG and nitrite (NO_2^-) {2}. The conversion of NO_2^- to NO occurs by an unknown mechanism {3}. An *S*-nitrosothiol is produced by a non-enzymatic reaction between NO and a thiol (probably cysteine or glutathione) {4}. Activation of guanylate cyclase by *S*-nitrosothiol {5} results in increased cGMP levels {6}. Smooth muscle relaxation then occurs {7}.

the hemoglobin-free isolated perfused rat liver and in GTN-tolerant rats. They demonstrated that GTN is metabolized to nitrite and GDN with simultaneous depletion of hepatic glutathione. In addition, they showed that livers depleted of glutathione fail to metabolize GTN. Studies in our laboratory with isolated perfused rat liver demonstrated that GSSG is released into the bile during GTN infusion [10]. This observation suggested that depletion of hepatic glutathione might be explained by its release into the bile following its oxidation during GTN metabolism [10].

The present study was undertaken to extend our investigation by examining the requirement for glutathione *S*-transferase and glutathione in the metabolism of GTN by cultured smooth muscle cells (SMC). These are the target cells in the clinical use of GTN.

METHODS

Cells. SMC were prepared from rat renal arteries by methods similar to those used for isolation of smooth muscle cells from aortic tissue [11]. Specifically, the vessel was removed and rinsed several times in Hanks' balanced salt solution (HBSS) buffered with 10 mM HEPES, pH 7.4, containing antibiotics. The vessel was opened and the luminal side was scraped with a scapel to remove the layer of endothelial cells. The vessel was then cut into pieces approximately 1–2 mm in diameter and placed luminal side down on a Petri dish. Enough medium was added to cover the piece of tissue. A small piece of sterilized screen was placed on top of each piece of tissue to prevent it from moving about and tearing the cells loose from the dish. In about 2 days, smooth muscle cells began to migrate out and divide so that they could be seen at the periphery of the tissue. After 7–10 days, the tissue could be removed without disturbing the underlying cells. The cells were freed by treatment with trypsin and plated in appropriate size Petri dishes in RPMI-1640 medium containing 10% supplemented calf serum and antibiotics (penicillin, 100 U/mL; streptomycin, 100 μg/mL; amphotericin, 0.25 μg/mL). Cells used in the experiments were identified as smooth muscle in origin by positive staining with antibodies made against smooth muscle actin, the characteristic smooth muscle cell morphology of "hills and valleys", and the development of post-confluent nodules [12].

The cells were maintained in RPMI-1640 medium plus serum and antibiotics in 100 mm Petri dishes (P-100). The cells were grown to confluency (approximately 2.3×10^6 cells/plate) before use. Prior to each experiment the medium was removed and the cells were rinsed with phosphate-buffered saline (PBS) to remove any remaining RPMI medium; then 5 mL of PBS containing 0.49 mM $MgCl_2$, 0.55 mM $CaCl_2$ and 5.6 mM glucose (PBS+) was added to each plate. After the addition of GTN, the plates were incubated in 5% CO_2 at 37° for the desired length of time. At the conclusion of the experiment, the PBS+ medium was removed and assayed for nitrite and total glutathione content. A 1-mL aliquot of 10% trichloroacetic acid was added to the cells and the cells were freed from the plate with a rubber policeman. The acidified cell suspension was centrifuged and the acid supernatant was neutralized with 0.3 M Na_2HPO_4. Total glutathione (GSH + GSSG) was determined in the neutralized solution [13]. Two P-100 plates were used per treatment in each experiment.

Cytotoxicity experiments. Cytotoxicity of GTN, indocyanine green (ICG), phorone and buthionine sulfoximine (BSO) to SMC was assessed using a ^{51}Cr release assay as previously described [14, 15]. Cultures of SMC were grown to confluency in 24-well cluster dishes and then incubated with ^{51}Cr (1 μCi/mL buffered HBSS, sp. act. 330 mCi/mg Cr) for 2 hr at 37°. The cells were rinsed twice with buffered HBSS, and GTN or the other compounds were added at the maximum concentrations used in the experiments described. After 1 hr, the incubation medium was removed (0.5 mL) and the cells were rinsed twice with 0.5 mL of buffered HBSS. The rinses were then combined with the incubation medium and the amount of ^{51}Cr released from the cells was determined by liquid scintillation counting. The amount of radioactivity remaining in the cells was determined after disruption with 1 N NH_4OH. The ^{51}Cr released from the cells was determined by

Table 1. Glutathione *S*-transferase activity of rat arterial SMC

Substrate	Concn (mM)	Specific activity* nmol conjugate formed / mg protein · min
1-Chloro-2,4-dinitrobenzene	1	56 ± 17 (7)
1,2-Dichloro-4-nitrobenzene	1	0† (6)
1,2-Epoxy-3-(*p*-nitrophenoxy)propane	0.5	24 ± 11 (6)
Ethacrynic acid	0.2	10 ± 5 (6)

* Values are means ± SD. The number of separate experiments is given in parentheses.
† Value measured was not significantly different than baseline value.

calculating the ratio of the amount of ^{51}Cr radioactivity in the supernatant divided by the combined radioactivity measured in the supernatant and in the cells. The amount of ^{51}Cr released from untreated controls was measured as an index of spontaneous release. ^{51}Cr released from treated cells was not significantly different from untreated controls, indicating that the compounds used in this study were not cytotoxic at the concentrations used (data not shown). Cells were observed by light microscopy before and after the incubation period to determine if there was morphological evidence for cell damage or detachment. Cell damage or detachment would be expected to produce an increase in the amount of ^{51}Cr in the medium. There was no evidence for cell damage or detachment as judged by light microscopy.

Assays. Glutathione *S*-transferase activity was measured as described by Habig *et al.* [13]. Glutathione was determined by the GSSG reductase-5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) recirculating assay of Tietze [16]. A standard curve was constructed daily. Nitrite was measured using the colorimetric assay originally described by Bell *et al.* [17]. An aliquot of the medium was acidified with 0.1 M HCl, and the sample was diazotized with sulfanilic acid. *N*-(1-Naphthyl)-ethylenediamine was added to the solution to form the azo dye. The absorbance was measured at 548 nm. A standard curve was constructed using sodium nitrite. The extent of nonenzymatic nitrite production was measured in boiled cells incubated with GTN in 5 mL PBS+. After 1 hr at 37°, the amount of nitrite produced was 3.5 to 7.5% of that produced by the same amount of GTN incubated with viable cells. Non-enzymatic nitrite formation was considered to be negligible and no correction for it was made.

Chemicals. Reduced glutathione, glutathione reductase, ICG, phorone, BSO, *N*-(1-naphthyl)-ethylenediamine and monoclonal antibodies to smooth muscle actin were purchased from the Sigma Chemical Co., St. Louis, MO. NADPH was obtained from the U.S. Biochemical Corp., Cleveland, OH. Sulfanilic acid was purchased from the Aldrich Chemical Co., Milwaukee, WI. ^{51}Cr (sp. act. 330 mCi/mg) was obtained from New England Nuclear, Boston, MA. RPMI 1640, HBSS and antibiotics for cell culture were purchased from GIBCO, Grand Island, NY. Supplemented calf serum was obtained from HyClone, Logan, UT. All other chemicals were of reagent grade.

RESULTS

Characterization of cultured SMC. The presence of glutathione *S*-transferase was sought in cultured SMC. Activity in SMC was determined with several substrates. It was greatest with 1-chloro-2,4-dinitrobenzene (Table 1). While measurable, the activities with 1,2-epoxy-3-(*p*-nitrophenoxy)propane and ethacrynic acid were only slightly above baseline. No activity was detectable with 1,2-dichloro-4-nitrobenzene. Although the specific glutathione *S*-transferase isoforms present have not been characterized further, the substrates which showed activity are metabolized primarily by transferases 1-1,2-2,3-3 and 3-4 [18]. GTN is metabolized by all of these isoforms, with transferase 3-4 showing the greatest activity [19]. Microsomal glutathione *S*-transferase shows activity with 1-chloro-2,4-dinitrobenzene; however, its activity with the other substrates tested is minimal [20].

Cellular glutathione content in SMC averaged 20.7 ± 7.9 nmol glutathione equivalents/10^6 cells (N = 23, range 10.6 to 37.8 nmol glutathione equivalents/10^6 cells). This is similar to glutathione values measured in other isolated cell systems. For example, endothelial cells have been reported to contain 7 nmol glutathione equivalents/10^6 cells [21] while isolated hepatocytes contain 45 nmol glutathione equivalents/10^6 cells [22]. Glutathione turnover in SMC was determined by treatment with BSO. BSO is an inhibitor of γ-glutamylcysteine synthetase and thus inhibits cellular glutathione synthesis [23]. Figure 2 shows the time course of glutathione depletion in cells pretreated with 0.44 and 0.88 mM BSO. The half-life of SMC glutathione under these conditions was 4.5 hr. This is a relatively rapid turnover of glutathione compared with that measured in other tissues [24].

The ability of SMC to metabolize GTN was assessed by measurement of nitrite production following GTN treatment. Nitrite production increased with increasing GTN concentration and with incubation time (Fig. 3).

Dependence of GTN metabolism on glutathione. The enzymatic nature of GTN metabolism to nitrite was examined by enzyme inhibition studies. ICG is a non-substrate inhibitor of the glutathione *S*-transferases [25]. SMC were incubated with concentrations of ICG ranging from 1 to 100 μM for 30 min prior to the addition of 500 nmol GTN. Nitrite production decreased with increasing ICG

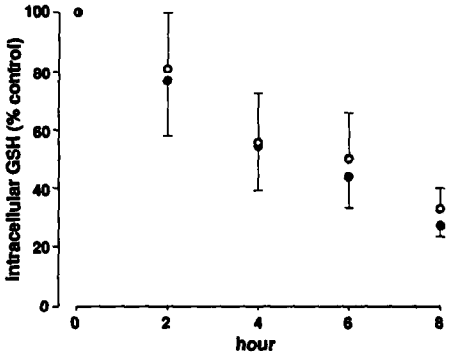

Fig. 2. Glutathione turnover in SMC. BSO [0.44 mM (○); 0.88 mM (●)] was added to confluent SMC cells in P-100 plates. Intracellular glutathione content of BSO-treated cells is expressed as a percentage of that measured in untreated cells at the same time. The initial intracellular glutathione content was 41.1 ± 17.3 nmol glutathione equivalents/10^6 cells. Each point is the average of at least six experiments (two plates per treatment per time point). One SD is shown by the error bar.

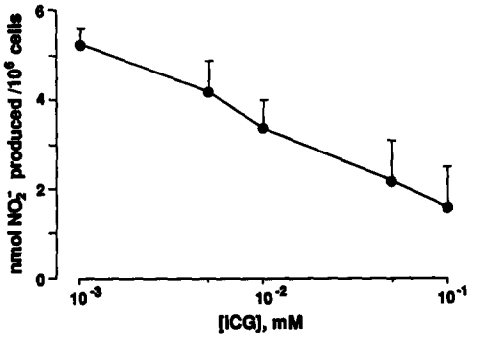

Fig. 4. Effect of ICG pretreatment on GTN-induced nitrite release in SMC. SMC were treated with the specified concentration of ICG for 30 min prior to the addition of 100 μM GTN. Nitrite (NO_2^-) released to the medium was measured 1 hr after GTN addition. Each point is the mean of four experiments (two plates per treatment per experiment). One SD is shown by the error bar.

concentration (Fig. 4). This suggests that the glutathione *S*-transferases in SMC metabolize GTN.

GTN metabolism was assessed in the presence of another glutathione *S*-transferase substrate, phorone. Phorone is conjugated with glutathione by glutathione *S*-transferase, resulting in the depletion of cellular glutathione [26]. Incubation of SMC with 0.3 to 2.6 mM phorone for 30 or 60 min caused a decrease in cellular glutathione to 7.6 ± 3.4 nmol glutathione equivalents/10^6 cells (N = 27). Subsequent addition of GTN (500 or 1000 nmol) to the cells for a 1-hr incubation resulted in a further decrease in cellular glutathione levels (2.3 + 2.6 nmol glutathione equivalents/10^6 cells (N = 27)). The amount of nitrite produced was proportional to the amount of glutathione present at the time of GTN addition (Fig. 5). Since phorone and GTN are both conjugated with glutathione by the glutathione *S*-transferases, they compete for metabolism. Thus, the decreased metabolism of GTN in the presence of phorone could be caused either by glutathione depletion or competitive inhibition of the glutathione *S*-transferases or by a combination of the two.

To examine the effect of decreased cellular glutathione on the metabolism of GTN by SMC, cells were pretreated with BSO. An 18-hr BSO pretreatment of SMC in RPMI 1640 markedly decreased cellular glutathione; however, nitrite production was not decreased a comparable amount (Table 2). A decrease of more than 85% in cellular glutathione resulted in a 35% decrease in nitrite production. Therefore, the possibility that other thiols might influence nitrite production was considered. Cysteine was removed from the cell

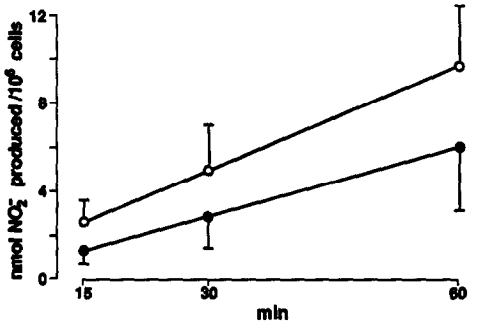

Fig. 3. GTN-induced nitrite production in SMC. GTN [50 (●) or 100 (○) μM] was added to SMC in PBS+ and incubated at 37° for the indicated time. Nitrite (NO_2^-) released to the medium was measured at the designated time after GTN addition. Each point is the mean of triplicate experiments (two plates per treatment per experiment). One SD is shown by the error bar.

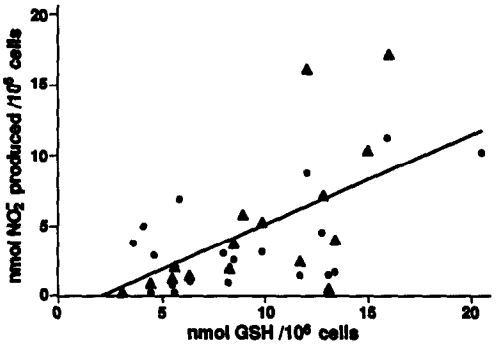

Fig. 5. GTN-induced nitrite production vs glutathione concentration in phorone-treated SMC. SMC were treated with 0 to 2.56 mM phorone for 30 or 60 min to deplete intracellular glutathione. After phorone pretreatment, the cells were rinsed with PBS and fresh PBS+ was added to the cells. GTN [0.1 mM (●) or 0.2 mM (▲)] was added to the cells, and the nitrite (NO_2^-) released to the medium was measured 1 hr after GTN addition. Nitrite released is plotted versus the intracellular glutathione content measured prior to GTN addition. Each point is the average of two plates per treatment. The linear regression line has a correlation coefficient (*r*) of 0.60.

Table 2. Effect of glutathione depletion on GTN metabolism*

Pretreatment	Glutathione (%)	Nitrite produced (%)
None	100	100
0.22 mM BSO†	14 ± 5	67 ± 9
0.22 mM BSO − cysteine‡	<1	32 ± 9

* SMC were incubated in RPMI 1640 with the specified pretreatment for 18 hr before the addition of GTN (0.1 mM). Values are expressed as percent of control value measured in SMC incubated in RPMI 1640 for the same time without any pretreatment. Intracellular glutathione content of untreated SMC was 21.0 ± 5.4 nmol glutathione equivalents/10^6 cells and 12.7 ± 3.4 nmol of nitrite was produced per 10^6 cells.

† N = 3.

‡ N = 7.

culture medium in combination with BSO addition during the 18-hr pretreatment. This resulted in the depletion of cellular glutathione to less than 1% of that measured in complete RPMI medium. Glutathione *S*-transferase activity was not affected by the removal of cysteine from the medium (data not shown). When these cells were incubated with GTN, nitrite production was only 32% of control (Table 2). Thus, depletion of cellular glutathione in combination with removal of cysteine from the culture medium resulted in a decreased metabolism of GTN by SMC. The finding that nitrite production was not eliminated completely by glutathione and cysteine removal leaves open the possibility that a non-thiol-dependent mechanism is responsible for GTN metabolism in this thiol-depleted state.

DISCUSSION

Previously, it has been reported that GTN metabolism in the liver occurs via a glutathione-dependent pathway [9, 10]. More clinically relevant, however, is the action of GTN on the vascular smooth muscle. This study shows that GTN is metabolized in cultured SMC by a glutathione-dependent pathway. Specifically, the glutathione *S*-transferases and glutathione are involved in GTN metabolism by the SMC. The glutathione dependence of GTN metabolism in cultured SMC makes it an appropriate model for evaluating the hypothesis that GTN tolerance is related to glutathione depletion.

Needleman *et al.* were the first to suggest that cellular sulfhydryl groups are involved in GTN metabolism [27]. Glutathione is the major non-protein thiol present in cells and is required for the metabolism of GTN by glutathione *S*-transferase [4, 5]. Our studies show that depletion of cellular glutathione or inhibition of glutathione *S*-transferase resulted in decreased GTN metabolism. Tolerance results from repeated and/or continuous GTN administration [28, 29]. Therefore, tolerance consisting of decreased GTN metabolism might result from the depletion of cellular glutathione by the continuous metabolism of GTN in the SMC. Oxidized glutathione formed during GTN metabolism (Fig. 1) is transported out of the cell if its concentration exceeds the capacity of glutathione reductase. Repletion of cellular glutathione would be expected to result in renewed GTN metabolism. The rapid turnover of SMC glutathione reported here could explain the rapid recovery from tolerance that is seen when a drug-free interval is included in the GTN treatment regimen [28, 30].

The involvement of glutathione *S*-transferase in GTN metabolism is well established. However, it appears that another mechanism may also be present in the SMC which can produce nitrite from GTN. Support for a glutathione-independent mechanism of GTN metabolism is provided by experiments in which cellular glutathione was decreased without affecting glutathione *S*-transferase activity. When glutathione was decreased by BSO pretreatment, nitrite production was decreased but it was not eliminated (Table 2). Nor was nitrite production eliminated by the removal of glutathione and cysteine. Thus, the possibility that another GTN metabolic pathway exists must be considered. These results provide the justification for evaluating the role of glutathione-dependent GTN metabolism in GTN tolerance.

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