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Tolerance to Nitroglycerin in Rabbit Aorta

INVESTIGATING THE INVOLVEMENT OF THE MU ISOZYME OF GLUTATHIONE S-TRANSFERASES

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ABSTRACT. We have proposed that glutathione S-transferases (GSTs), especially the mu isozyme, play a critical role in the metabolism of nitroglycerin (glyceryl trinitrate, GTN), leading to pharmacologic effects. Here we study this enzyme(s) during tolerance development in male New Zealand white rabbits. Each aorta was divided into two segments designated as GTN pretreated and buffer control. Tolerance was induced in rabbit aortic strips so assigned by incubation with GTN (0.22 mM). The activity of the mu isozyme and of total GSTs was determined in portions of each segment. In each rabbit aorta, the response to GTN (0.5 μM) was determined in GTN-pretreated and buffer-pretreated strips by measuring cyclic GMP levels (N = 7 pairs) and percent relaxation (N = 4 pairs). In GTN-pretreated strips, a significant decrease was observed in the activity of the mu isozyme of GST, while the total GST activity was unchanged as compared with control strips. The decrease in isozyme activity correlated very well with the decrease in response to GTN. Two rabbit aortae did not become tolerant, and the activity of the mu isozyme was also not affected. The levels of thiols were not affected by GTN pretreatment and aortae tolerant to GTN did not develop tolerance to S-nitroso acetylpenicillamine (SNAP), indicating that thiol depletion and guanylate cyclase desensitization probably play a minor role in tolerance development to GTN in our model. These studies suggest that tolerance to GTN in rabbit aorta *in vitro* is associated with a decrease in GST mu activity, which correlates well with the decrease in GTN response. BIOCHEM PHARMACOL 51;10:1357–1363, 1996.

KEY WORDS. glyceryl trinitrate; cGMP; relaxation; TSO; CDNB

Several publications describe the existence of nitrate tolerance in humans and in experimental animals *in vivo* [1–6]. These findings prompted the investigation of the possible existence of tolerance in isolated vascular smooth muscle taken from animals made tolerant to organic nitrate esters *in vivo* [7, 8]. Although *in vivo* tolerance to organic nitrates could be a complex combination of pharmacokinetic alterations, physiologic alterations (such as neurohormonal compensatory responses), and biochemical alterations, *in vitro* tolerance determined in isolated vascular tissues is probably the direct result of biochemical alterations in the vascular smooth muscle cells by organic nitrates.

In the past, various hypotheses have been proposed to describe tolerance to GTN‡ at a cellular level. These have included a reduction in thiol groups or thiol depletion [9–12], reduced guanylate cyclase activity due to a desensitization of this enzyme [13–16], and reduced vascular biotransformation of the drug [17–23].

The vasodilating action of GTN is linked intimately to its metabolism to the dinitrate metabolites. It has been shown that the time course of dinitrate formation from GTN in vascular segments closely parallels that of vasodilation and that *in vitro* tolerance to GTN results in a significantly reduced level of dinitrates in the aortic wall, suggesting reduced biotransformation of GTN in the vascular wall [17]. Reduced levels of metabolites of both GTN and isosorbide dinitrate in tolerant rabbit aortic strips and a significant cross-tolerance between GTN and isosorbide dinitrate mediated effects on metabolite concentrations within the vascular wall have been reported [23]. Such an effect has also been demonstrated in cell cultures of vascular and non-vascular origin [18]. Based upon these studies, it has been suggested that the decreased biotransformation could be due to a change or a decrease in the activity of the enzyme systems involved in metabolizing these organic nitrates or, alternatively, to co-factor depletion.

GSTs and cytochrome P450s [24] have been proposed to be involved in the metabolic bioactivation of GTN. We have demonstrated previously that in rabbit aorta the cGMP increase and tissue relaxation are diminished in the presence of a GST inhibitor, ethacrynic acid [25]. Furthermore, a good correlation was observed between the GST mu activity in different rabbit aortae and the cGMP increase in response to GTN [26]. It has also been demon-

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‡ *Abbreviations:* GTN, glyceryl trinitrate; GST, glutathione S-transferase; SNAP, S-nitroso acetylpenicillamine; PE, 1-phenylephrine hydrochloride; CDNB, 1-chloro-2,4-dinitrobenzene; TSO, *trans*-stilbene oxide, and cGMP, cyclic 3′,5′-guanosine monophosphate.

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strated that there is a good correlation between GTN metabolism and GST mu activity using anti-serum against the mu and pi isozymes [27, 28]. Therefore, it seems reasonable to hypothesize that the reduced metabolism of GTN, observed in tolerance development, could be due, at least in part, to an alteration in the activity of the GST mu isozyme. The study described here was carried out to evaluate this hypothesis.

MATERIALS AND METHODS

GTN was purchased as 10 mL vials of Tridil® from DuPont Pharmaceuticals (Wilmington, DE). PE, glutathione, CDNB, cysteine, *N*-acetylcysteine and TSO were obtained from the Sigma Chemical Co. (St. Louis, MO). ^{3}H-Labeled TSO was purchased from American Radiolabelled Chemicals, Inc. (St. Louis, MO). SNAP was obtained from Research Biochemicals International (Natick, MA). The cGMP radioimmunoassay kit was purchased from Amersham (Arlington Heights, IL). Hexanol and 2-(4-maleimidophenyl)-6-methyl benzothiazole were purchased from Aldrich (Milwaukee, WI). 2-(4-Maleimidophenyl)-6-methyl benzothiazole was recrystallized from chloroform prior to use. Acetonitrile and methanol were HPLC grade and purchased from Fischer (Santa Clara, CA).

Most of the techniques utilized here followed procedures from our laboratory described previously [25, 29]. Briefly, aortae from 17 male New Zealand white rabbits (Nitabell Rabbitry, Hayward, CA) weighing 2–3 kg, were used in these studies (four rabbits for the dose–response studies with GTN and SNAP, seven rabbits for the studies comparing the enzyme activities and responses in tolerant and non-tolerant strips, three for the vehicle control experiments, and three for the thiol measurements). The rabbits were anesthestized using a subcutaneous injection of ketamine (40 mg/kg as 1.5 mL of a 100 mg/mL solution in Water for Injection, USP) and decapitated 15 min later. The thoracic cavity was exposed, and the descending aorta was removed surgically and immediately placed in a beaker containing Krebs buffer (NaCl, 119 mM; KCl, 4.8 mM; KH_2PO_4, 1.2 mM; $NaHCO_3$, 25 mM; $CaCl_2$, 2.5 mM; glucose, 11.1 mM), which was gassed continuously with carbogen (95% oxygen and 5% carbon dioxide). After isolation of the aorta, excess fat and connective tissues were removed carefully; during this process the buffer was constantly replaced with fresh buffer. The endothelium was not disturbed. The aorta was then helically cut and divided into two equal segments.

Enzyme Activity and GTN Response Studies

Each of the two segments was suspended in Krebs buffer contained in 25-mL jacketed circulating water tissue baths maintained at 37° and gassed with carbogen. One of the two segments was assigned as GTN pretreated (segment A), while the other as buffer preteated i.e. control (segment B).

GTN (0.22 mM) was added to segment A while the same volume of buffer was added to segment B. The two segments were allowed to stand for 1 hr. Then each segment was divided into two strips (A1, A2, B1, B2). One strip of each segment was frozen immediately between blocks of dry ice for enzyme assays to be performed later. The remaining strip was washed for 30 min (four times), and effect measurements were then performed on these strips.

Effect Measurements

After being washed for 30 min, each strip (both GTN pretreated and control) was treated with 0.5 μM GTN. At the end of 5 min, the strips were frozen using tongs precooled in liquid nitrogen and frozen immediately on dry ice. Analysis of cGMP was then carried out in each of these strips. In four of the seven rabbit aortae, the relaxation effect of GTN was also measured. The procedures used for measuring vasorelaxation were the same as described previously [25]. Briefly, the strips were precontracted using phenylephrine (approximately 0.3 to 0.6 μM), and the relaxation effects of GTN were recorded via transducers coupled to a Grass model 7 polygraph (Quincy, MA). These strips were then frozen for cGMP analysis together with strips upon which relaxation measurements were undertaken.

Analysis of cGMP Levels

cGMP levels were analyzed using the procedure previously described [25]. Briefly, the frozen strips were homogenized in 1 mL of 6% trichloroacetic acid to precipitate proteins; the homogenate was then centrifuged at 2200 *g* for 10 min at 2–4° to remove the protein precipitate. The supernatant was transferred into a fresh tube and washed with a 4-fold volume of diethyl ether saturated with water. This procedure was repeated at least four times. Each time the ether layer was carefully removed, after the tubes were vortexed, and allowed to stand for some time. After removing the residual ether by evaporation under a stream of nitrogen, the samples were then assayed for cGMP using a ^{3}H-labeled cGMP radioimmunoassay kit.

Enzyme Activity Measurements

The strips frozen for enzymatic analysis were homogenized using three times the volume of phosphate-buffered saline (pH 7.4) and spun at 2200 *g* for 5 min. The supernatants were then used for the enzyme assays.

Total GST Activity (CDNB Activity)

Total GST activity was measured using the standard procedure [30]. Briefly, the assay was carried out in a 3-mL plastic cuvette that contained 1 mM CDNB, 5 mM glutathione, 0.1 mM potassium phosphate buffer, pH 6.65, and 10 μL of the aortic supernatant preparation. The increase in absorbance was measured at 340 nm at room temperature. The extinction coefficient is 9.6 mM^{-1} cm^{-1} at 25°

[30], and one unit of activity was defined as 1 μmol/min. CDNB activities are expressed in μmol CDNB/min/mg protein.

GST mu Isozyme Activity (TSO Activity)

GST mu activity was measured using a radiometric assay similar to that previously described [31]. Briefly the aortic supernatant fraction (10 μL) was incubated in a final volume of 100 μL containing phosphate-buffered saline, pH 7.4, 4 mM glutathione and 250 μM [^{3}H]TSO (sp. act. = 15 Ci/mmol). The reaction mixture was incubated at 37° for 10 min, and the reaction was terminated by extraction with 2 vol. of hexanol. The percent radioactivity in the aqueous phase was calculated from the radioactivity measurements in the organic and aqueous phases using liquid scintillation counting. The non-enzymatic control rates of reaction, determined from inactivated boiled tissues, were subtracted from the experimentally determined rates to yield the reported activities as (nmol/min)/CDNB unit and (nmol/min)/mg protein.

Protein Assays

Protein was estimated using the classic procedure [32]. Protein concentrations were expressed in mg/mL.

Control Experiments

To test the potential effects of the cosolvent system in the formulation of GTN (30% propylene glycol and ethanol), aortae from three rabbits were divided as described in the GTN-pretreatment experiments. One segment was treated with buffer (same volume as for the GTN-pretreatment experiment), while the remaining segment was treated with an equal volume of a solution containing the cosolvents for 1 hr. One strip from each segment was frozen for enzyme assays, while the other was tested with GTN (0.5 μM) for relaxation.

Measurement of Thiol Levels in Tolerant Versus Non-Tolerant Aorta

The levels of thiols (glutathione and cysteine) in rabbit aortic tissues were determined using an HPLC analysis technique with fluorimetric derivatization and detection [33]. Briefly, 100 mg of aortic tissue, either treated with 0.22 mM GTN (N = 3) or treated with buffer control (N = 3), was homogenized using 20 mM EDTA in 40% acetonitrile. Pre-column derivatization was performed using 2-[4-maleimidophenyl]-6-methylbenzothiazole yielding fluorescent derivatives with excitation λ = 310 nm and emission λ = 405 nm. The following HPLC conditions were used:

Column: ODS 25 cm Beckmann column
Mobile phase: 10 mM KH_2PO_4, 0.1% hexane sulfonic acid, 35% acetonitrile and pH adjusted to 4.5.

Standard curves were made for standard solutions of glutathione and cysteine (0–10 nmol/mL) using *N*-acetylcysteine as an internal standard.

SNAP Response in GTN-Pretreated Aortae

Each aorta was divided into four equal strips, with each strip suspended in a separate tissue bath. Two strips were assigned as buffer-pretreated and two as GTN-pretreated strips. Strips 2 and 4 were treated with 0.22 mM GTN for 1 hr, while strips 1 and 3 were pretreated with an equal volume of buffer. After 1 hr, the strips were washed four times over a total period of 30 min and then contracted with a submaximal dose of PE. Strips 1 and 2 were then exposed to various concentrations of GTN, while strips 3 and 4 were exposed to various concentrations of SNAP. A dose–response curve was constructed, using the cumulative percent relaxation from PE-induced contraction of strips as the response measurement.

Data Analysis

Data are expressed as means ± SD. Statistical comparisons between the GTN-pretreated and control tissue enzyme activities, cGMP levels and percent relaxation in the different rabbit aortae were made using the paired *t*-test. Statistical significance was accepted as $P < 0.05$.

RESULTS

cGMP Levels in GTN-Pretreated Versus Control Segments

cGMP levels were measured in response to GTN (0.5 μM) in GTN-pretreated and control rabbit aortae *in vitro*. In our previous studies with rabbit aortic strips, we observed that this concentration of GTN produces significant increases in cGMP levels. In segments that were pretreated with buffer control for 1 hr, cGMP levels averaged 50.2 ± 11.0 pmol/g wet weight (N = 7), while the segments pretreated with a high concentration of GTN (0.22 mM) for 1 hr had lower cGMP levels of 26.4 ± 12.2 pmol/g wet weight (statistically significant, paired *t*-test, $P < 0.05$, $N = 7$, Fig. 1).

Two of these seven rabbit aortae showed resistance to the development of tolerance to GTN (GTN-pretreated/control ratio for cGMP response was > 0.9), with mean cGMP levels of 44.2 pmol/g net weight in non-tolerant segments vs 40.3 pmol/g wet weight in tolerant segments (Fig. 2B). In the remaining five rabbit aortae, a significant difference was observed in the cGMP levels (Fig. 2A) upon GTN pretreatment (52.6 ± 11.9 pmol/g wet weight in control vs 20.9 ± 8.8 pmol/g wet weight in GTN-pretreated segments, N = 5).

There was a large variability in the extent of tolerance development in the various rabbit aortae. That is, the GTN-pretreated/control ratio for cGMP response varied from 0.18 to 0.93. No statistically significant difference was found in the cGMP levels between the control rabbit aortae

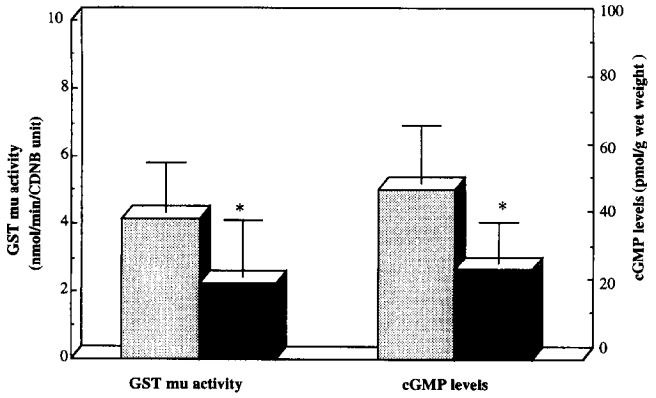

FIG. 1. cGMP levels in response to 0.5 μM GTN and the GST mu activity in GTN-pretreated (▨) and control (▩) aorta (N = 7). Values are means ± SD. Key: (*) $P < 0.05$.

that were pre-contracted with phenylephrine (N = 4, 53.8 ± 13.3 pmol/g wet weight) for relaxation measurements and those that were directly treated with GTN (N = 3, 45.3 ± 5.9 pmol/g wet weight).

Percent Relaxation in GTN-Pretreated Versus Control Segments

The percent relaxation in response to 0.5 μM GTN was measured in four of the seven rabbit aortae studied. In the segments that were pretreated with buffer control for 1 hr, an 89.9 ± 7.6% relaxation to GTN was observed, while a 47.0 ± 25.8% relaxation was observed in the segments pretreated with GTN (0.22 mM) for 1 hr (Fig. 3). As observed with the cGMP levels, a considerable variability, ranging from 0.22 to 0.78, was observed in the GTN-pretreated/control ratios for the relaxation responses to GTN. The GTN-pretreated strips that exhibited a low relaxation response to 0.5 μM GTN compared with their paired controls

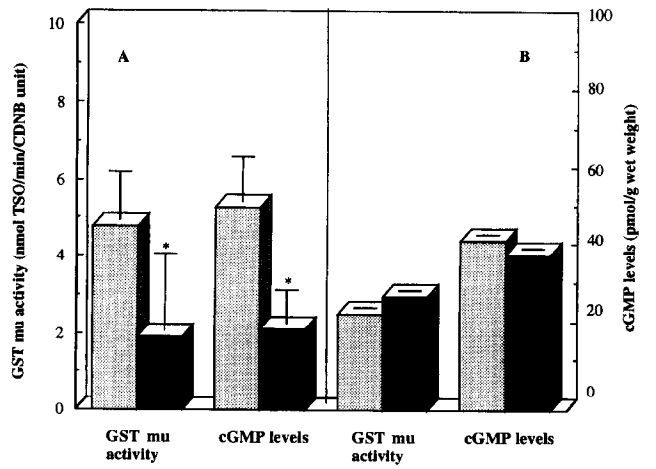

FIG. 2. Resistance to the development of tolerance in rabbit aorta. Shown are cGMP levels in response to GTN and the GST mu activity in rabbits pretreated with GTN (▨) and in control (▩) aorta. (A) Results in five rabbit aortae exhibiting tolerance. Values are means ± SD. Key: * $P < 0.05$. (B) Results in two rabbit aortae not exhibiting tolerance.

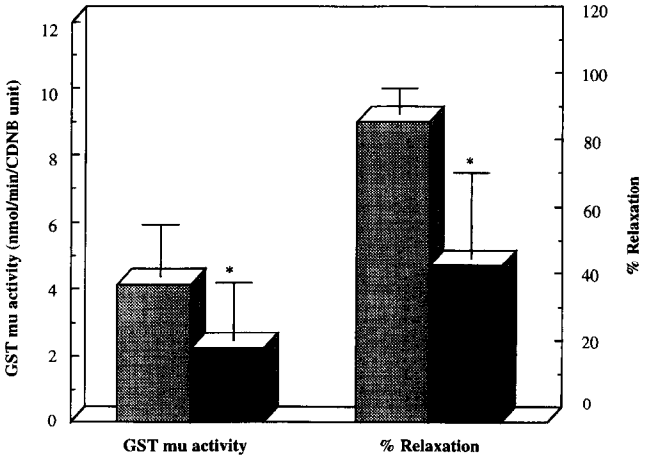

FIG. 3. Percent relaxation in response to 0.5 μM GTN and the GST mu activity in GTN-pretreated (▨) and control (▩) rabbit aortae (N = 4). Values are means ± SD. Key: (*) $P < 0.05$.

also showed low cGMP levels compared with their controls. There was a good correlation (r^2 = 0.778) between relaxation and cGMP levels in the GTN-pretreated and control rabbit aortae (Fig. 4), where two pairs of measurements were made in the four aortae.

Total GST Activity in Rabbit Aorta During Tolerance to GTN

Total GST activity in the segments pretreated with GTN (0.22 mM), 0.32 ± 0.06 μmol CDNB/min/mg protein, was not significantly different from the total GST activity in the segments that were pretreated with same volume of buffer, 0.34 ± 0.10 μmol CDNB/min/mg protein. Additionally, protein concentrations were not significantly different in the GTN-pretreated segments (3.23 ± 1.09 mg/mL of aortic homogenate) versus the control segments (3.55 ± 0.83 mg/mL of aortic homogenate).

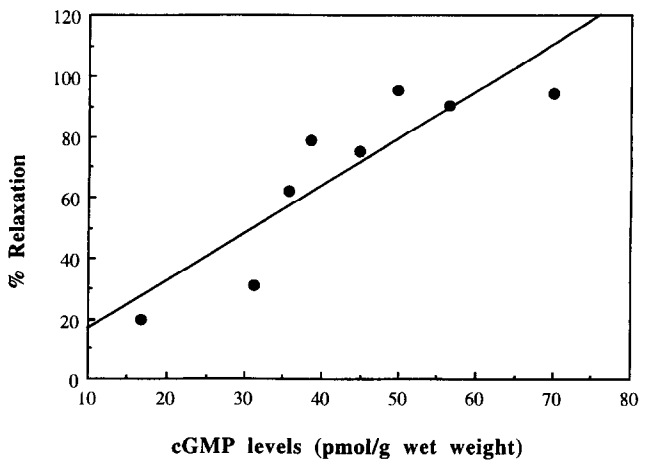

FIG. 4. Correlation (r^2 = 0.778) of cGMP levels and percent relaxation in GTN-pretreated and control rabbit aortae. Both measurements were made pre- and post-GTN treatment in four pairs of aortae.

GST mu Isozyme Activity in GTN-Pretreated Aortae

Activity of the mu isozyme of GST (normalized to total GST) was significantly lower in the segments that were pretreated with GTN (0.22 mM), 2.22 ± 1.71 nmol TSO/min/CDNB unit as compared with those pretreated with buffer, 4.12 ± 1.53 nmol TSO/min/CDNB unit (Fig. 1). GST mu activity in two of the seven rabbit aortae studied remained unchanged (average activity of 2.95 vs 2.51 nmol TSO/min/CDNB unit in GTN-pretreated segments vs control, respectively; Fig. 2B). In the remaining five aortae, the mu isozyme activity in the GTN-pretreated segments was significantly lower than the control segments (4.76 ± 1.28 nmol TSO/min/CDNB unit in control vs 1.92 ± 1.97 nmol TSO/min/CDNB unit in GTN-pretreated, N = 5, Fig. 2A). On an equiprotein basis, GST mu activity in GTN-pretreated aortic strips was 61% of the GST mu activity in control strips (1.25 nmol TSO/min/mg protein in control vs 0.76 nmol TSO/min/mg protein in GTN-pretreated segments of aortae).

Correlation of Effect Measurements to Activity Measurements

On the average, a 40% decrease in the GST mu activity was observed in the seven rabbit aortae, i.e. the mean GTN-pretreated/control ratio for the GST mu isozyme activity was 0.6. This correlated very well with the GTN-pretreated/control ratio of 0.55 for the cGMP response to 0.5 μM GTN (Fig. 1, right-hand side). Furthermore, the GTN-pretreated/control ratio for relaxation response to 0.5 μM GTN was 0.53, which also correlated well with the GTN-pretreated/control ratio for GST mu activity, i.e. 0.6 (Fig. 3). Furthermore, upon closer inspection, it was seen that of the seven rabbit aortae, two seemed to be resistant to becoming tolerant, and the GST mu activities in those two rabbit aortae also remained unchanged (Fig. 2B). On the other hand, the remaining five rabbits became tolerant (as demonstrated by the 60.2% decrease in cGMP levels), and a corresponding decrease (59.6%) was also observed in the GST mu activity (Fig. 2A).

Control Experiments

No significant differences (paired *t*-tests, N = 3) were observed in the percent relaxation (106 ± 26 vs 109.4 ± 23%) or in the GST mu activity (4.90 ± 0.60 vs 5.52 ± 0.76 nmol TSO/min/CDNB unit) in strips treated with buffer versus cosolvents, respectively.

Levels of Thiols in Tissues Pretreated with GTN

The levels of glutathione and cysteine were measured in aortic tissues pretreated with GTN and compared with those treated with a buffer control. Glutathione concentration in the GTN-pretreated strips was 0.82 ± 0.18 μmol/g wet weight compared with 0.70 ± 0.07 μmol/g wet weight in the control strips. Cysteine concentration in the GTN-pretreated tissue was 0.12 ± 0.03 μmol/g wet weight compared with 0.11 ± 0.01 μmol/g wet weight. There was no significant difference (paired *t*-test) between the levels of the two thiols in GTN-pretreated versus control aortic strips.

Response to SNAP in Tissues Pretreated with GTN

The potencies of GTN and SNAP in the rabbit aortic strips were comparable ($EC_{50\ GTN} = 1.07 \times 10^{-7}$ M and $EC_{50\ SNAP} = 2.14 \times 10^{-7}$ M). Strips of rabbit aorta that were pretreated with a high dose of GTN did not show a significantly decreased response to SNAP (Fig. 5), while aortic strips from the same rabbits showed significant tolerance to GTN. Upon fitting the dose–response data to a sigmoidal E_{max} model, no significant changes were observed in the parameters for SNAP pre- and post-GTN (0.22 mM) treatment (parameters not shown).

DISCUSSION

While tolerance to the hemodynamic effects of GTN is well known, the mechanism of tolerance development remains controversial. A reduced intracellular metabolic activation of GTN, either due to a decrease in the cofactors that are essential for the reaction or a decrease in the enzyme activity (or alteration of the crucial enzyme), has been proposed as a mechanism for tolerance development to GTN [17–23]. Other hypotheses have been proposed to explain the development of tolerance to GTN such as a decrease in the guanylate cyclase activity [13–16] or reduced intracellular thiol levels in the vascular smooth muscle cells [9–12].

We have demonstrated previously that GST mu activity correlates with GTN-mediated relaxation and cGMP levels in rabbit aorta [26]. Based on these previous results we

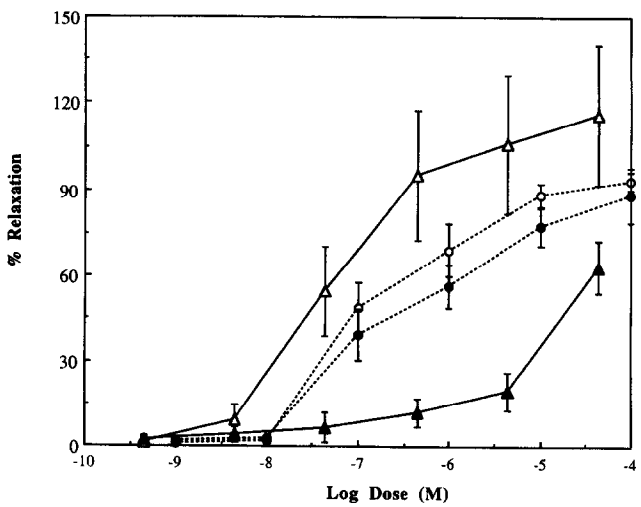

FIG. 5. Percent relaxation in response to GTN in GTN-pretreated (—▲—) and control aortic strips (—△—) and SNAP in GTN-pretreated (—●—) versus control strips (—○—). Values are means ± SD, N = 4.

hypothesized that the tolerance development to GTN may be associated with a decrease in GST mu activity. To examine the validity of this hypothesis we have induced tolerance in rabbit aorta *in vitro* and studied the changes in GST activity as compared with the extent of tolerance (measured using cGMP levels and relaxation). Each rabbit aorta serves as its own control; thus, GTN tolerance could be examined on a pairwise basis at the site of action, using a biochemical marker of response such as cGMP formation.

We found that the GST mu isozyme activity was reduced significantly in tolerant tissues versus the non-tolerant tissues. This is consistent with the observations of Chern *et al.* [27], who found that there was about a 50% decrease in GST mu activity upon induction of GTN tolerance *in vivo*. The total GST activity did not change upon pretreatment with GTN. This indicates a specific alteration of the mu isozyme activity only, and that the mu isozyme represents a relatively small portion of total GST (~15% [28]) so that a significant decrease in mu activity may be obscured in total GST measurements. We compared the reduced enzyme activities to the reduced response to GTN in the rabbit aorta. The levels of cGMP in the GTN pretreated and control rabbit aortae in response to GTN compared well with percent relaxation. Two of the seven rabbits exhibited less than a 10% decrease in response to GTN after the GTN pretreatment, suggesting resistance to the development of tolerance. As predicted here, the GST mu isozyme activity in those two rabbits also did not decrease. In the remaining five rabbits, the decrease in the GST mu activity corresponded well with the decrease in cGMP levels upon tolerance induction (~60% decrease in both).

The similar decreases in cGMP levels and GST mu activity that we observed suggest that the decreased response to GTN could be a result of the decreased enzyme activity/level. However, it could also be a secondary effect of some other biochemical change in the vascular smooth muscle, which then produces a decrease in the enzyme activity. At this point our results do not differentiate between these two possibilities, and it is very difficult to determine if the second mechanism is occurring. However, it is reasonable to say that a decrease in GST mu isozyme may be a reason for the tolerance to GTN, based upon the good correlation observed between the response to GTN and the isozyme activity. Although there are many reports supporting the involvement of GSTs in the vasoactivity of GTN, this is not consistent across the literature. In a recent publication, Haefeli *et al.* [34] reported that the GST mu polymorphism does not explain the variability in GTN responsiveness, and Chung *et al.* [35] suggested that GSTs may not mediate the formation of nitric oxide from GTN in microsomes from bovine coronary artery smooth muscle. The reason for this inconsistency is not clear but could be due to measurement of a different endpoint in humans, which may be influenced by other factors (*in vivo*). The results of Chung *et al.* [35] cannot be compared due to a species difference.

To examine thiol depletion as a possible mechanism for the decrease in GST mu activity, concentrations of glutathione and cysteine were measured in aortic tissues pretreated with GTN and compared with their buffer controls. No differences between the levels of thiols in GTN-pretreated or control tissues were observed. This suggests that for our *in vitro* model of tolerance, thiol depletion does not occur and that the decrease in isozyme activity that we observed in tolerant tissues was not due to a decrease in thiol cofactors, but is probably a direct result of inactivation of the isozyme.

To examine the possibility of guanylate cyclase desensitization as a possible mechanism of tolerance to GTN, we studied the effect of GTN pretreatment (0.22 mM) on responses to SNAP (a compound that does not need to be enzymatically metabolized). No cross-tolerance to SNAP was observed, indicating that guanylate cyclase desensitization could not have led to the decreased responses to GTN in our model. Furthermore, these experiments also rule out the possibility that the high GTN concentration may have affected guanylate cyclase, leading to the decreased responses. Vehicle controls performed suggest that the vehicle in the GTN formulation did not have any effect on the enzyme activity or the response to GTN.

To summarize, our studies have shown that GST mu isozyme activity is reduced in GTN-tolerant tissues and that this decrease correlates well with the decrease in the effect of GTN, as measured by cGMP levels and percent relaxation. Overall in our *in vitro* model, GTN tolerance in rabbit aortic strips was accompanied by an inactivation of GST mu isozyme. These results point towards a possible role of GST mu in the bioactivation of and tolerance development to GTN. The relevance of such a decrease in enzyme activity to the clinical setting remains to be established. Such *in vitro* tolerance mimicked by incubation with high concentrations of GTN may not reflect the tolerance development *in vivo* in humans or may be one of the possible causes. *In vivo* tolerance may also include compensatory mechanisms, e.g. changes in renin and catecholamines.

Decreased enzyme activity could occur via a suicidal mechanism by which the enzyme is inactivated by a metabolite that is formed via enzyme catalysis. Evidence for a metabolite induced inhibition of the enzyme systems involved has been presented by Cossum and coworkers [36, 37]. Such a metabolite inhibition may also be operative in degradation/elimination reactions and could possibly explain the reduced plasma clearance and accumulation of metabolite after infusion of organic nitrates *in vivo* [38–40]. This mechanism requires further probing and will be investigated in our laboratory.

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