Biochemical Pharmacology, Vol. 43, No. 10, pp. 2247–2254, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

EFFECTS OF SULFOBROMOPHTHALEIN AND ETHACRYNIC ACID ON GLYCERYL TRINITRATE RELAXATION

DAVID T-W. LAU and LESLIE Z. BENET*
Department of Pharmacy, School of Pharmacy, University of California, San Francisco, CA 94143-0446, U.S.A.

(*Received* 9 *February* 1991; *accepted* 17 *January* 1992)

Abstract—The effects of sulfobromophthalein (SBP) and ethacrynic acid (ECA), both inhibitors of glutathione *S*-transferase (GST), or glyceryl trinitrate (GTN)-induced vasorelaxation were investigated in rabbit aortic strips. The aortic strips were pre-contracted with phenylephrine, followed by relaxation with 0.5 μM GTN, with or without 0.1 mM SBP or ECA. ECA was observed to inhibit GTN relaxation approximately 32%, whereas SBP did not alter the GTN activity. The dinitrate metabolites (GDN) of GTN in the tissues were also measured. The amounts of both GDNs were decreased in the ECA-treated, but not the SBP-treated group. Moreover, in the ECA-treated group, a strong correlation was obtained between the loss of GTN activity and the decrease in GTN metabolism. Concentration–response studies also revealed that ECA attenuates GTN relaxation. The slope factor of the concentration–response curves was decreased by ECA, but not by SBP, although both inhibitors caused a mild decrease in E_{max}. In the 9000 *g* supernatant of rabbit aorta, ECA was also observed to inhibit GTN metabolism more significantly than SBP. The results suggest that the mechanism of GTN activation may involve a GST isozyme that possesses high activities towards ECA.

Glyceryl trinitrate (GTN†), more commonly known as nitroglycerin, is a potent vasodilator that is frequently prescribed as an anti-anginal agent. Although it was first used in the 19th century, its mechanism of action remains controversial. It is generally believed that GTN and other vasodilators act as prodrugs [1], which are metabolized rapidly by vascular smooth muscle cells in the blood vessels to generate pharmacologically active species that can elicit vasorelaxation, rather than acting directly as receptor-binding ligands [2].

Confirmation of which enzyme(s) is important in metabolizing GTN and other organic nitrates within the blood vessels has not been universally agreed upon. It has been reported that glutathione *S*-transferases (GST) can metabolize GTN in the liver [3]. Recent work from our laboratory has also shown that hepatic GTN metabolism is glutathione dependent [4]. Moreover, various GST inhibitors were shown to be able to block hepatic GTN degradation in the cytosolic fraction of rabbit livers [5]. GST consists of a family of isozymes that are present in various organs in the body [6]. It is possible that the GSTs which exist in the blood vessels can be responsible for GTN metabolism in those tissues. In addition, development of nitrate tolerance has been linked to a decrease in the extent of nitrate metabolism in various *in vitro* studies [7–9]. Therefore, it is essential to decipher the role of GSTs in the vascular metabolism of organic nitrates. Here, the effects of two known substrate inhibitors for GSTs—sulfobromophthalein (SBP) and ethacrynic (ECA)—on GTN relaxation were investigated in aortic strips pre-contracted with phenylephrine. The metabolism of GTN, as assessed by the formation of its dinitrate metabolites, 1,2- and 1,3-glyceryl dinitrates (1,2-GDN and 1,3-GDN), was also examined in order to probe possible changes in GTN metabolism caused by these inhibitors.

MATERIALS AND METHODS

GTN was purchased as 10-mL vials of Tridil® from DuPont Pharmaceuticals (Wilmington, DE). 1,2- and 1,3-GDN (>99% purity) standards were supplied by Marion Laboratories (Kansas City, MO). SBP, ECA and *l*-Phenylephrine hydrochloride (PE) were obtained from the Sigma Chemical Co. (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).

New Zealand White rabbits (Nitabell Rabbitry, Hayward, CA; male, 2–3 kg) were anesthetized with an intramuscular injection of ketamine (40 mg/kg) and decapitated 15 min later. The thoracic cavity of the animal was immediately opened, and the descending aorta was removed quickly from the body. Adipose and connective tissues were removed, and the aortic tissue was immersed in a beaker of Krebs buffer (NaCl, 119 mM; KCl, 4.8 mM; KH_2PO_4, 1.2 mM; $MgSO_4$, 1.2 mM; $NaHCO_3$, 25 mM; $CaCl_2$, 2.5 mM; glucose, 11.1 mM), bubbled continuously with carbogen (95% oxygen, 5% carbon dioxide). The tissue was then cut into three helical strips of dimensions 3 cm × 4 mm. The strips were

* Corresponding author. Tel. (415) 476-3853; FAX (415) 476-0688.

† Abbreviations: GTN, glyceryl trinitrate; GST, glutathione *S*-transferase; SBP, sulfobromophthalein; ECA, ethacrynic acid; 1,2-GDN, 1,2-glyceryl dinitrate; 1,3-GDN, 1,3-glyceryl dinitrate; and PE, *l*-phenylephrine hydrochloride.

individually suspended in a buffer medium contained in a jacketed circulating water bath maintained at 37°. One of these strips was assigned as the control, and the remaining two as the inhibitor-treated strips. The contraction and relaxation of the tissues were recorded via transducers that were coupled to a Grass model 7 polygraph (Quincy, MA). The tension on the strips was maintained at 1 g. The tissues were allowed to equilibrate for at least 30 min before addition of any chemicals.

Upon stabilization, 100 μM PE was added to contract the strips maximally. The strips were then washed four to six times, 3 min apart, with the buffer. This was then followed by two longer washes for 15 min, and the strips were then allowed to stabilize. PE (100 μM) was added for a second time, and the maximal tension achieved by each strip was recorded. Interestingly, the second PE addition always resulted in a greater contraction than the first. The strips were then washed as described following the first PE addition. Upon stabilization, the strips were treated with consecutive additions of 0.1 μM PE, until the tension reached 60–80% of the maximal contraction previously recorded (in most cases, 0.3 to 0.5 μM PE was necessary). At this time one of the three strips was selected randomly as a control, while the other two were incubated for at least 10 min with inhibitors SBP or ECA at final concentrations of 0.1 mM. GTN was then added to each strip at a concentration of 0.5 μM, and the relaxation was recorded at 0.5, 1, 2, and 5 min. At 5 min, the strips were taken out of the tissue baths, wrapped in a piece of aluminium foil, dipped into a jar of liquid nitrogen, weighed, and stored at −70° until assay.

Amounts of GDN metabolites were assayed as reported previously [10], with slight modifications. Briefly, the strips were extracted three times with 10-mL mixtures of pentane and methyl-*t*-butyl ether (8:2). The organic extract was evaporated under a nitrogen stream, and reconstituted in 50 μL *n*-butyl acetate. *o*-Iodobenzyl alcohol was used as the internal standard. A 0.2-μL portion of the reconstituted sample was injected into a Varian 6000/6500 gas chromatograph equipped with an Ni-63 ECD detector (Varian, Sugar Land, TX). The detection limit of the assay for the GDNs is 0.1 ng, and the amounts of GDNs measured were normalized to strip weights for comparison purposes.

For the concentration–response studies, one of the three strips was randomly assigned to be the control, and the other two strips were incubated with SBP or ECA at two different inhibitor concentrations (low: 0.01 mM, or high: 0.1 mM). The strips were allowed to stabilize for 10 min. Different concentrations of GTN were then added to the strips, from approximately 0.1 nM to 4 μM (ten different concentrations), in an increasing order. The relaxation of the strips caused by each of the GTN additions was recorded, and a concentration–response curve was obtained. A sigmoidal E_{max} model was used to fit the concentration–response relationship. The fitted parameters—E_{max}, EC_{50}, and the Hill coefficient (γ)—were then compared among the control and the inhibitor-treated groups.

For the metabolism studies in homogenates, fifteen rabbit aorta were homogenized with 3 vol. of phosphate buffer. The blood vessel homogenates were centrifuged at 9000 *g* and the supernatant fraction was decanted, saved, and incubated in a water bath with the temperature maintained at 37°. GSH (2 mM) was added as the co-factor. 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 30 and 60 min, and were frozen immediately in a mixture of dry ice and methanol. The concentrations of GTN and GDNs were determined as previously described. Due to the presence of nonenzymatic GTN degradation in phosphate buffer, a vial containing just the phosphate buffer and GSH was incubated with GTN as the control. The extent of non-enzymatic GDN formation at each sampling time was subtracted from the observed metabolism in the experimental samples.

For statistical comparisons, the repeated measures ANOVA was used. Dunnett's test was performed in comparing the two treatment groups to the control. For the correlation studies, linear regression was performed using geometric mean analysis, which assumes that error may exist in both the x- and y-axes measurements [11]. The correlation coefficient was tested for significance using the *t*-test.

RESULTS

No difference in the extents of PE-induced contraction in the three groups of aortic strips was

Table 1. PE-induced contraction and GTN-induced relaxation in control, SBP-treated, and ECA-treated groups at 5 min

Treatments	PE-induced contraction (g)	GTN-induced relaxation (g)	% Relaxation	% Relaxation expressed as a % of control
Control (C)	2.43 ± 0.81	2.05 ± 0.70	83.5 ± 7.8	100
SBP-treated (S)	2.57 ± 0.83	2.12 ± 0.75	81.9 ± 7.0	98.2 ± 4.1
ECA-treated (E)	2.59 ± 0.62	1.51 ± 0.61	56.6 ± 13	67.8 ± 15
Statistics	C = S = E	C = S > E	C = S > E	S > E

Values are means ± SD, N = 9.

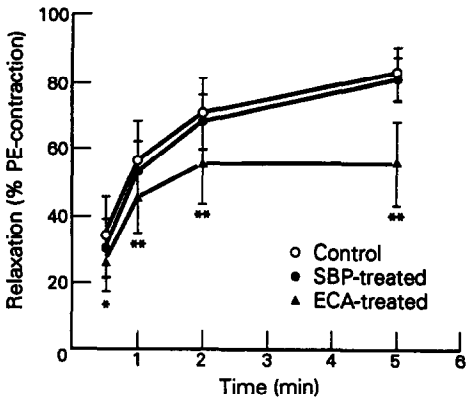

Fig. 1. Time course of relaxation, as percentages of PE-induced contraction, in control, SBP-treated, and ECA-treated groups. Values are means ± SEM, N = 9; Key: (*) $P < 0.05$ and (**) $P < 0.01$ vs control. See Table 1 for control values.

observed (Table 1, column 2). In addition, the contraction states of the aortic strips were not altered upon addition of the inhibitors (data not shown). However, the ECA-treated group possessed a significantly ($P < 0.05$) lower response to GTN than the control and the SBP-treated groups after 5 min of GTN incubation, as depicted in Table 1. The time courses of relaxation during the first 5 min after GTN addition for the three treatments are shown in Fig. 1. For the ECA-treated strips, significant attenuation in the relaxation response was initially observed at 30 sec, and the effect was increasingly evident up to 5 min. There were no detectable differences between the SBP-treated strips and the control at any time point.

The amounts of the GDN metabolites of GTN in the aortic strips at the end of the 5-min incubation were measured and are shown in Table 2. Considerable variability in these values was observed. Using repeated measures ANOVA, no statistically significant difference between the SBP-treated and the control strips was found. However, there were significant reductions in the amount of total GDNs, as well as that of 1,2-GDN, in the ECA-treated strips, when compared to the control group, indicating a decreased extent of GTN metabolism. The amount of 1,3-GDN seems to be decreased as well, although the difference was not statistically significant when compared to the control ($P = 0.066$), probably due to the variability observed. When the amounts of GDNs in the inhibitor-treated groups were normalized to the amount in the control for each animal, significant reductions in the formation of both GDNs were observed in the ECA-treated group, as shown in Table 2. For the SBP-treated group, no statistically significant difference in the formation of either GDN was observed. To identify a possible correlation between the attenuation of GTN effects and the reduction in GTN metabolism for the ECA-treated group, the relaxation caused by GTN addition was plotted against the formation of GDNs, each as a percentage

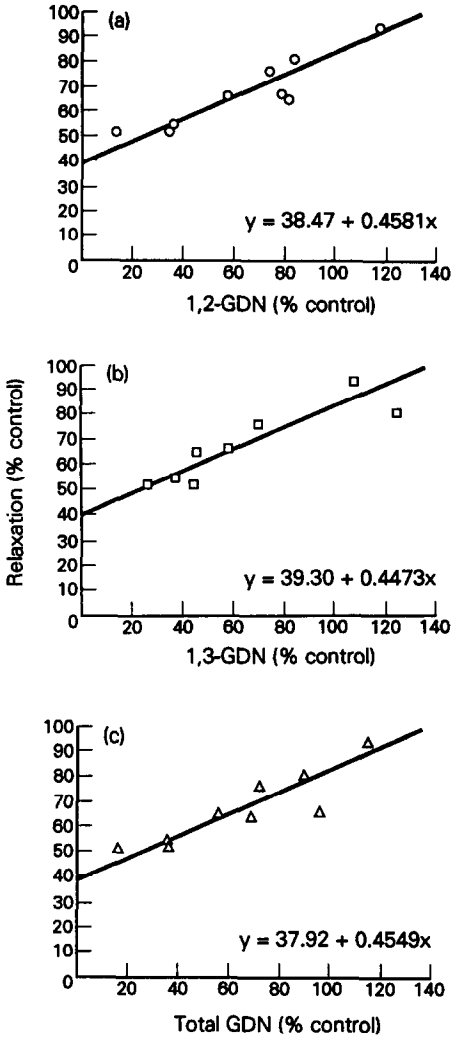

Fig. 2. Relationship in the presence of ECA between the aortic strip relaxation and the weight-normalized formation of: (a) 1,2-GDN ($r^2 = 0.848$), (b) 1,3-GDN ($r^2 = 0.805$), and (c) total GDN (1,2-GDN + 1,3-GDN) ($r^2 = 0.809$). All values are presented as the percentages of the corresponding control values. N = 9, except in (b), where one set of data was ignored because the 1,3-GDN measurement was below detection limits. See Table 2 for control values.

of the control value. Strong correlations were found between the GTN effects and the formation of 1,2-GDN ($r^2 = 0.848$, $P < 0.01$), 1,3-GDN ($r^2 = 0.805$, $P < 0.01$), and the sum of the two GDNs ($r^2 = 0.809$, $P < 0.01$), as illustrated in Fig. 2a, b and c, respectively.

To demonstrate that the effects of the inhibitors did not exist only for a particular concentration of GTN, concentration–response relationships for GTN-induced relaxation were obtained in the presence and absence of SBP and ECA, each at two different concentrations (0.01 and 0.1 mM), as shown in Fig. 3a and b. It is obvious that ECA gave a more pronounced change in the concentration–response

Table 2. 1,2- and 1,3-GDNs, expressed as amount (normalized by the weight of the strips), or as the percentage of the control, in SBP- and ECA-treated strips

	Control	SBP-treated	ECA-treated
1,3-GDN (ng/g tissue)	10.9 ± 11.7	8.2 ± 6.6	6.9 ± 6.6
1,2-GDN (ng/g tissue)	24.9 ± 11.5	26.7 ± 10.2	16.5 ± 10.3*
Total GDN (ng/g tissue)	35.8 ± 22.0	34.9 ± 13.9	23.5 ± 15.5*
% 1,3-GDN (% control)	100	81 ± 19	64 ± 35*
% 1,2-GDN (% control)	100	119 ± 48	64 ± 32*
% Total GDN (% control)	100	111 ± 39	66 ± 32*

Values are means ± SD, N = 9.
* $P < 0.05$ vs control.

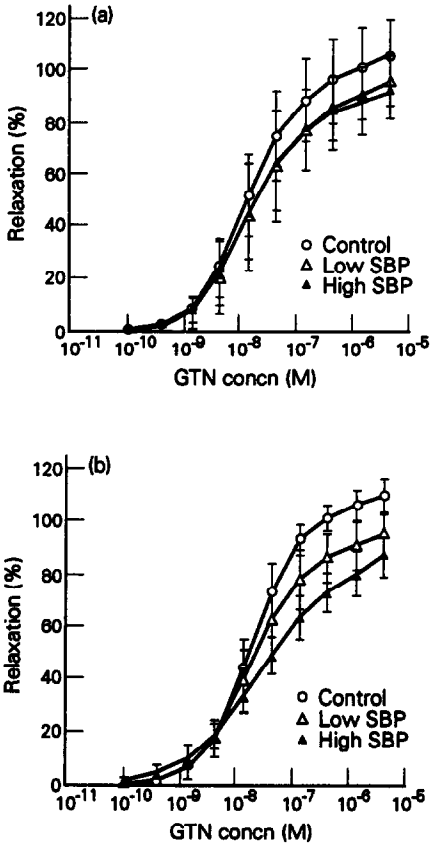

Fig. 3. Concentration–response curves of rabbit aortic strips to GTN-induced relaxation. Tissues were treated with either low (0.01 mM) or high (0.1 mM) concentrations of (a) SBP (N = 10) or (b) ECA (N = 8), and were compared to the control in each study. Values are means ± SD.

relationship, especially at the higher inhibitor concentration, whereas SBP caused only minor alterations in the concentration–response relationship to GTN. The parameters obtained from a Hill equation fit to the concentration–response curves are listed in Table 3. A mild but significant decrease in the E_{max} (~10%), with no change in the EC_{50} or the Hill coefficient was observed with the SBP-treated groups. With the ECA-treated strips, a similar magnitude of change in E_{max} was observed (Table 3). However, there was also a significant reduction in the slope factor (γ) of the Hill equation. In addition, the magnitude of alteration seemed to increase as the concentration increased, indicating a concentration-dependent inhibition of the effects of GTN on the aortic strips by ECA. Although considerable variability was observed in the EC_{50} values of both inhibitor-treated groups, the EC_{50} values of the 0.1 mM ECA-treated group almost achieved statistically significant differences relative to the control ($P = 0.059$).

To compare the effects of these inhibitors on homogenized tissues versus the aortic strips, the 9000 *g* fraction of a pool of fifteen rabbit aorta was incubated with 40 ng/mL GTN in the presence and absence of these inhibitors. The reported concentrations of GDNs corrected for non-enzymatic GTN degradation (in a vial containing GTN and GSH only) are depicted in Fig. 4. The changes in total GDN concentrations were more prominent in the ECA- than the SBP-treated group at both the 30- and 60-min time points, consistent with the more extensive inhibition of GTN metabolism and effects observed with the ECA-treated group in the aortic strip studies.

DISCUSSION

It has been proposed that GTN and other organic nitrates exert their action via biotransformation to pharmacologically active species such as nitrosothiols [1] or nitric oxide [12–14]. It has been shown that GTN metabolism is coupled to cGMP elevation and vasorelaxation [15, 16] in aortic strips. Tolerance to organic mitrates was also found to be associated with an attenuation of nitrate metabolism in tolerant tissues [7–9]. It is generally believed that the metabolism of nitrates in vascular smooth muscle cells is carried out enzymatically, although the enzyme responsible for the activation of these agents has not been identified. It has been suggested that GST may play a role in this activation process [17]. Recently, glutathione-dependent enzymatic activities, including those of peroxidase, reductase, and transferase, have been reported in human arteries and veins [18].

Previously, it was observed that hepatic GTN

Table 3. Parameters obtained from fitting the GTN concentration–response curves of rabbit aortic strips to the Hill equation in control, SBP- or ECA-treated strips, at two different inhibitor concentrations

	E_{max} (% contraction)	EC_{50} (nM)	Hill coefficient (γ)
SBP studies (N = 10)			
Control	104 ± 15.3	19.3 ± 12.0	0.987 ± 0.128
SBP-treated (0.01 mM)	94.5 ± 10.1	39.7 ± 50.9	0.995 ± 0.115
SBP-treated (0.1 mM)	91.2 ± 11.1	27.7 ± 29.6	0.963 ± 0.139
ANOVA	P < 0.01	NS	NS
ECA studies (N = 8)			
Control	108 ± 4.1	23.6 ± 10.3	1.033 ± 0.079
ECA-treated (0.01 mM)	93.9 ± 8.1	30.1 ± 31.7	0.903 ± 0.080
ECA-treated (0.1 mM)	90.0 ± 9.9	56.8 ± 59.1	0.637 ± 0.080
ANOVA	P < 0.001	NS	P < 0.001

Values are means ± SD.

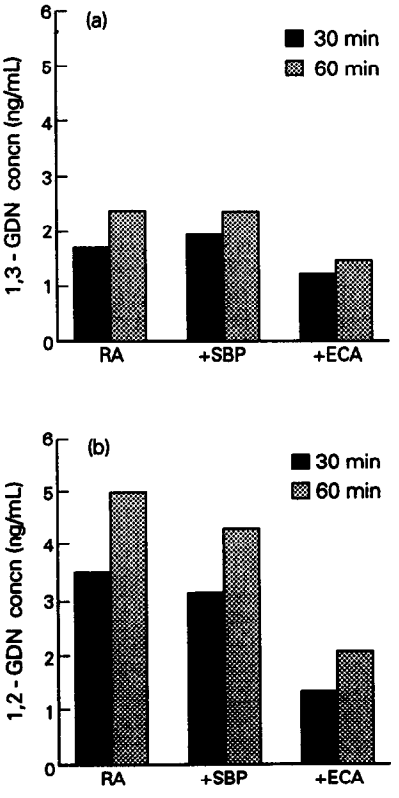

Fig. 4. (a) 1,3-GDN and (b) 1,2-GDN recovered (corrected for nonenzymatic metabolism) from 40 ng/mL GTN incubations in the 9000 *g* supernatant of rabbit aorta. Key: RA: control; +SBP: RA with 0.02 mM SBP; +ECA: RA with 0.02 mM ECA. Results are the means of duplicate values, measured at 30 or 60 min after GTN addition.

metabolism is a glutathione-dependent process [4], which can be inhibited by various GST substrates [5]. There is also evidence that more than one GST isozyme may be involved, and that isozymes may exhibit different regioselectivities for the denitration reaction of GTN. Recently, we found that a cytosolic GSH-dependent, enzymatic process responsible for GTN metabolism was present in bovine coronary artery homogenates.* In addition, the rate of GTN metabolism was reduced significantly by two GST substrate-inhibitors—ECA and SBP. If GSTs are indeed involved in vascular GTN metabolism, these enzymes may also have a role in mediating nitrate-induced relaxation. In this study, using the rabbit aortic strip model, a well-established *in vitro* model for studying the pharmacological actions of vasoactive compounds, the possible involvement of GST in the action of GTN was investigated by examining the effects of two substrate inhibitors (ECA and SBP) on GTN metabolism and its pharmacological action.

We observed here that ECA addition led to attenuation of GTN-induced relaxation in all the aortic strips tested, although considerable variability in the extent of reduction was observed. GTN-induced relaxation upon ECA treatment, as a percentage of the control value, ranged from 51.4 to 94.7%. However, this variability also makes it possible to examine the correlation between the reduction in GTN metabolism and its attenuated effect over a range of values. The strong correlations found suggest that ECA inhibits GTN metabolism, which leads to a reduction in the formation of GDNs and the pharmacologically active species, which in turn leads to attenuation in GTN effects. The fact that there are positive y-intercepts in Fig. 2 is not surprising, since the time courses of dinitrate formation are not likely to match exactly those relating to relaxation. The significant change in the slope factor of the concentration–response relationship of GTN following ECA addition also suggests an alteration in the kinetics of the formation of vasoactive intermediates from GTN in the presence of ECA. Moreover, GTN metabolism in

* Lau DTW, Chan EK and Benet LZ, Glutathione *S*-transferase mediated metabolism of glyceryl trinitrate in subcellular fractions of bovine coronary arteries. Manuscript submitted for publication.

the 9000 *g* supernatant fraction of the rabbit aorta was also inhibited by ECA, supporting the hypothesis that the crucial enzyme for GTN metabolism and activity may belong to a GST which exhibits high activity to ECA.

The results here agree with previous *in vitro* experiments [19, 20] where ECA was shown to attenuate the effects of GTN and other vasodilators. However, it cannot be concluded that ECA inhibits GTN-induced relaxation solely via inhibition of GST activity towards GTN. In fact, it can be interpolated from Fig. 2c that if there is no reduction in GTN metabolism (i.e. GDN formation is 100% of control values), one can observe a 16.6% decrease in the capacity of the aortic strips to be relaxed by GTN (i.e. the y-axis intercept for 100% on the x-axis is 83.4%). This suggests that ECA may also affect some other steps in the process of GTN activation. Rapaport and Murad [20] have shown that 0.1 mM ECA does not affect either the cGMP level or guanylate cyclase activities in rat aorta. However, ECA was shown to be able to inhibit sodium nitroprusside-induced relaxation of aortic strips. It was suggested that the inhibitor could affect certain unspecified sulfhydryl groups that are not contained within guanylate cyclase. It is possible that some of the effects of ECA may be related to this non-specific phenomenon. However, most of the preparations (7 out of 9) demonstrated 20% or more reduction in GTN-induced relaxation, suggesting that this explanation cannot account for all the GTN activity that was lost upon ECA treatment. Moreover, the strong correlation between the decrease in GTN metabolism and its effect suggests that inhibition of GTN metabolism is an essential mechanism by which ECA inhibits the pharmacological effects of GTN. It can also be argued that the glutathione pool in the aortic strip may be depleted. It has been shown that depleting glutathione in blood vessels by pretreatment with buthionine sulfoximine does not cause any change in the sensitivity of rabbit aortic strips towards GTN [21], suggesting that glutathione is normally present at an excess concentration. In addition, if these inhibitors serve only as sulfhydryl depletors, one would expect GTN effects to be antagonized to a certain extent in the SBP-treated strips, unless the two inhibitors differ vastly in the rates of their metabolism. Rather, we believe that the lack of inhibition of GTN effects by SBP indicates that the attenuation in GTN effects for ECA-treated strips probably results from a specific inhibition of a certain enzymatic pathway, instead of non-specific depletion of co-factors.

It should be noted that the results here contradict those of an earlier publication [21], where it was reported that GTN-induced relaxation could be antagonized by SBP. It is not clear why different results were found in this work. However, there is a noticeable difference in the sensitivity of the aortic strips to GTN relaxation. In this study, more than 80% relaxation was observed at 5 min after GTN addition, whereas the earlier report showed only about 35% relaxation with the same GTN concentration, although the time of measurement was not specified. The difference in the dose–response relationship is apparently caused by the difference in the method of eliciting vasoconstriction before GTN addition. In the earlier study, KCl was used to elicit vasoconstriction, whereas in this study, phenylephrine was used. It could be possible that SBP was not taken up significantly from the buffer medium in this experiment, whereas in the earlier study, its uptake was enhanced by the KCl-depolarized tissues. Moreover, the mechanisms of KCl- and PE-induced contraction may be different so that a higher concentration of GTN may be needed to antagonize the contraction state of the tissues. It is not clear whether the resultant differences in the baseline GTN concentration–response curves will affect the results of the inhibitor studies. However, it can be reasoned that since the EC_{50} for GTN relaxation reported in this study is much lower, the tissues should be more sensitive to any inhibition by the inhibitors. Moreover, GTN concentration–response studies were also performed in the presence of SBP. There was no change in the EC_{50}, and only a slight change in the E_{max}—approximately 10%. In addition, the extent of SBP inhibition of GTN metabolism in the rabbit aorta homogenate was found to be much less than that for ECA. Therefore, we believe that the effect of SBP on GTN relaxation, if there is any, is much less pronounced than the effect of ECA observed here. Recently, another group of investigators also observed a lack of effect of SBP on bovine coronary rings with respect to GTN action [22]. The results of our study imply that the crucial GST isozyme involved in vascular GTN metabolism may possess a higher activity towards ECA than SBP.

GST is known to exist as three major classes of isozymes—alpha, mu, and pi [23]. One limitation of this study is the cross-reactivities of the inhibitors to individual isozymes of GSTs. For example, SBP was known to be a relatively specific substrate and inhibitor for the mu-isoform; however, at higher micromolar concentrations, it has also been shown to inhibit the alpha-form [23]. Similarly for ECA, which has been regarded as a GST-pi substrate, it was demonstrated recently that ECA is a better inhibitor to mu-isozymes [24, 25]. Therefore, it is difficult to determine the identity of the GST isozyme which is involved in GTN metabolism. However, it can be concluded that the GST present in rabbit aorta probably exhibits a higher activity to ECA than SBP. Recently, Chern *et al.* [26] have found that in tissues made tolerant to GTN both *in vitro* or *in vivo*, 1-chloro-2,4-dinitrobenzene (CDNB) activity, a substrate for all GST isozymes, was not different from that in nontolerant control tissues. However, the activities to metabolize *trans*-stilbene oxide (TSO) and GTN were markedly reduced to the same extent—approximately 30% for tolerant tissues prepared *in vitro* and 50% for such tissues *in vivo*. Since TSO is a GST-mu specific substrate, it can be implied that the mu-isoform may be responsible for the action of nitrates. Tsuchida *et al.* [27] recently reported the isolation and purification of GST isozymes in human aorta which exhibit GTN activities. Some of those GST isozymes were found to exhibit immunoreactivity to anti-mu serum [27]. However, the GTN-metabolizing activity of this

purified isozyme can also be inhibited by SBP. Since the preparations used in that study consisted of purified fractions of GST isozymes, it is not surprising that the ability of SBP to inhibit GTN metabolism was more prominent than what was observed in this study. However, in determining the specific activities of the five class mu forms of GST isozymes, two of the five isozymes isolated by Tsuchida *et al.* [27] were found to exhibit high activities towards ECA. One of these isozymes (pI = 8.3) was also shown to exhibit high activities to GTN. On the other hand, none of these class mu isozymes exhibited activities towards conjugating SBP. It is possible that the inhibitory effects on GTN metabolism by ECA in these purified enzyme preparations may even be more extensive than that for SBP, although this was not examined.

Investigation of the mechanism of GTN metabolism in blood vessels is essential for the understanding of both the drug's mechanism of action, as well as the development of tolerance, a well-observed clinical phenomenon for organic nitrates. It has been shown that the extent of GTN metabolism decreases in aortic strips that have developed tolerance to the drug. The role of GSTs in tolerance development of organic nitrates warrants further investigation. In summary, it has been demonstrated that ECA diminished both GTN relaxation and GTN metabolism, resulting in a good correlation between the decrease in relaxation and the extent of GTN metabolism, as measured by GDN formation. In contrast, SBP did not seem to affect GTN metabolism and the pharmacological effects in rabbit aorta. It can be concluded that the enzyme which is crucial for nitrate metabolism as well as the scheme of action of these agents possesses activities towards ECA. The results from this study, together with the recent reports on the role of GSTs in vascular GTN metabolism, suggest that GST and, in particular, the class mu isozymes, may be the crucial enzyme involved in the vascular activation of GTN and other organic nitrates.

Acknowledgements—This work was supported in part by NIH Grant HL32243. The authors thank Marion Laboratories, Kansas City, MO, U.S.A., for supplying pure samples of the dinitrate metabolites, and Ms. Elaine K. Chan for her excellent technical assistance.

REFERENCES

1. Ignarro LJ, Lippton H, Edwards JC, Baricos WH, Kadowitz PJ and Gruetter CA, Mechanism of vascular smooth muscle relaxation by organic nitrates, nitrites, nitroprusside, and nitric oxide: Evidence for the involvement of *S*-nitrosothiols as active intermediates. *J Pharmacol Exp Ther* **218**: 739–749, 1981.
2. Kawamoto JH, Brien JF, Marks GS and Nakatsu K, Mechanism of glyceryl trinitrate-induced vasodilation. II. Lack of evidence of specific binding of GTN to bovine pulmonary vein. *J Pharmacol Exp Ther* **244**: 328–334, 1988.
3. Kamisaka K, Habig WH, Ketley JN, Arias IM and Jakoby WB, Multiple forms of glutathione *S*-transferase and their affinity for bilirubin. *Eur J Biochem* **60**: 153–161, 1975.
4. Lau DT-W and Benet LZ, Differential formation of dinitrate metabolites from glyceryl trinitrate in subcellular fractions of rabbit liver. *Biochem Pharmacol* **38**: 543–546, 1989.
5. Lau DTW and Benet LZ, Nitroglycerin metabolism in subcellular fractions of rabbit liver: Dose dependency of glyceryl dinitrate formation and possible involvement of multiple isozymes of glutathione *S*-transferases. *Drug Metab Dispos* **18**: 292–297, 1990.
6. Corrigall AV and Kirsch RE, Glutathione *S*-transferase distribution and concentration in human organs. *Biochem Int* **16**: 443–448, 1988.
7. Fung H and Poliszczuk R, Nitrosothiol and nitrate tolerance. *Z Kardiol* **75** (Suppl 3): 25–27, 1986.
8. Bennett BM, Leitman DC, Schröder H, Kawamoto JH, Nakatsu K and Murad F, Relationship between biotransformation of glyceryl trinitrate and cyclic GMP accumulation in various cultured cell lines. *J Pharmacol Exp Ther* **250**: 316–323, 1989.
9. Slack CJ, McLaughlin BE, Brien JF, Marks GS and Nakatsu K, Biotransformation of glyceryl trinitrate and isosorbide dinitrate in vascular smooth muscle made tolerant to organic nitrates. *Can J Physiol Pharmacol* **67**: 1381–1385, 1989.
10. Lee FW, Watari N, Rigod JF and Benet LZ, Simultaneous determination of nitroglycerin and its dinitrate metabolites by capillary gas chromatography with electron capture detection. *J Chromatogr* **426**: 259–266, 1988.
11. Sokal RR and Rohlf FJ, *Biometry*, p. 547. W. H. Freeman, New York, 1981.
12. Katsuki S, Arnold W, Mittal C and Murad F, Stimulation of guanylate cyclase by sodium nitroprusside, nitroglycerin and nitric oxide in various tissue preparations and comparison to the effects of sodium azide and hydroxylamine. *J Cyclic Nucleotide Res* **3**: 23–25, 1977.
13. Feelisch M and Noack EA, Correlation between nitric oxide formation during degradation of organic nitrates and activation of guanylate cyclase. *Eur J Pharmacol* **139**: 19–30, 1987.
14. Slack CJ, McLaughlin BE, Nakatsu K, Marks GS and Brien JF, Nitric oxide-induced vasodilation of organic nitrate-tolerant rabbit aorta. *Can J Physiol Pharmacol* **66**: 1344–1346, 1988.
15. Brien JF, McLaughlin BE, Breedon TH, Bennett BM, Nakatsu K and Marks GS, Biotransformation of glyceryl trinitrate occurs concurrently with relaxation of rabbit aorta. *J Pharmacol Exp Ther* **237**: 608–614, 1986.
16. Brien JF, McLaughlin BE, Kobus SM, Kawamoto JH, Nakatsu K and Marks GS, Mechanism of glyceryl trinitrate induced vasodilation. I. Relationship between drug biotransformation, tissue cylic GMP elevation, and relaxation of rabbit aorta. *J Pharmacol Exp Ther* **244**: 322–327, 1988.
17. Kukovetz WR and Holzmann S, Mechanism of nitrate-induced vasodilation and tolerance on a biochemical base. *Z Kardiol* **75** (Suppl 1): 39–44, 1985.
18. Mezzetti A, Di Ilio C, Calafiore AM, Aceto A, Marzio L, Frederici G and Cuccurullo F, Glutathione peroxidase, glutathione reductase and glutathione transferase activities in the human artery, vein and heart. *J Mol Cell Cardiol* **22**: 935–938, 1990.
19. Moffat JA, Abdollah H, Rollwage D and Armstrong PW, Ethacrynic acid: Acute hemodynamic effects and influence on the *in vivo* and *in vitro* response to nitroglycerin in the dog. *J Cardiovasc Pharmacol* **7**: 637–642, 1985.
20. Rapaport RM and Murad F, Effects of ethacrynic acid and cystamine on sodium nitroprusside-induced relaxation, cyclic GMP levels and guanylate cyclase activity in rat aorta. *Gen Pharmacol* **19**: 61–65, 1988.
21. Yeates RA, Schmid M and Leitold M, Antagonism of glycerol trinitrate activity by an inhibitor of glutathione

S-transferase. *Biochem Pharmacol* **38**: 1749–1753, 1989.
22. Chung SJ and Fung H-L, Vascular metabolic activation of nitroglycerin to nitric oxide in microsomes appears to contribute to its relaxation activity. *Pharm Res* **8** (Suppl): S–305, 1991.
23. Mannervik B, Ålin P, Guthenberg C, Jensson H, Tahir MK, Warholm M and Jörnvall H, Identification of three classes of cytosolic glutathione transferase common to several mammalian species: Correlation between structural data and enzymatic properties. *Proc Natl Acad Sci USA* **82**: 7202–7206, 1985.
24. Ploemen JHTM, van Ommen B and van Bladeren PJ, Inhibition of rat and human glutathione *S*-transferase isoenzymes by ethacrynic acid and its glutathione conjugate. *Biochem Pharmacol* **40**: 1631–1635, 1990.
25. Hansson J, Berhane K, Castro VM, Jungnelius, Mannervik B and Ringborg U, Sensitization of human melanoma cells to the cytotoxic effect of melphalan by the glutathione transferase inhibitor ethacrynic acid. *Cancer Res* **51**: 94–98, 1991.
26. Chern WH, Serabjit-Singh CJ, Lanzo CA, Han BJ, Shaffer JE and Lee FW, The metabolism of nitroglycerin in rabbit aorta correlates with the activity of the mu class glutathione *S*-transferase, but not the appearance of nitric oxide. *FASEB J* **5**: A1220, 1991.
27. Tsuchida S, Maki T and Sato K, Purification and characterization of glutathione transferases with an activity toward nitroglycerin from human aorta and heart. Multiplicity of the human class Mu forms. *J Biol Chem* **265**: 7150–7157, 1990.