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Conversion of Nitroglycerin to Nitric Oxide in Microsomes of the Bovine Coronary Artery Smooth Muscle is Not Primarily Mediated by Glutathione-S-Transferases[1]

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ABSTRACT

The pharmacological action of organic nitrate vasodilators [*e.g.*, nitroglycerin (NTG)] is thought to be mediated through metabolic conversion to nitric oxide (NO); conversion leads to vasodilatation, whereas diminished conversion in chronic therapy may lead to pharmacological tolerance. The biochemical nature of this process, however, is poorly understood. Glutathione-S-transferases (GST) have been shown to metabolize organic nitrates in the liver, but it is not known whether these enzymes are involved in this pharmacologically relevant process. We, therefore, compared the activities of conversion of NTG to NO *vs.* those of GST in microsomal suspensions of bovine coronary artery smooth muscle tissue. A classical GST substrate, 1-chloro-2,4-dinitrobenzene, inhibited NO production in microsomes, suggesting possible involvement of GST in organic nitrate activation. However, GST activity derived from microsomes exhibited a different heat lability profile compared to that of NO generation. Known inhibitors of GST (*viz.*, indomethacin and bromosulfophthalein) did not alter the NO-generating activity in microsomes. Glutathione was a critical cofactor for GST, but not for NO generation from NTG, and thiols other than glutathione (*e.g.*, N-acetyl-L-cysteine and thiosalicylic acid) also could facilitate NO production. Moreover, comparison to a commercially available purified liver GST preparation showed that, at the same GST activity toward 1-chloro-2,4-dinitrobenzene, the microsomal incubation produced about 8 times more NO than the purified liver GST. Radiation inactivation analysis of the functional molecular sizes of GST and the NO-producing enzyme(s) suggested that the enzymes were of different molecular weights (54 kD and 160 kD, respectively). Gel filtration chromatography showed that the GST and NO-producing activities were dissociated, and that the respective molecular weight estimations (49 and 210 kD) were consistent with those found by radiation inactivation. These data, therefore, strongly indicated that GST are not primarily responsible for the conversion of organic nitrates to NO in microsomes of the bovine coronary artery smooth muscle.

The organic nitrates, such as NTG, are important therapeutic agents used for the treatment of a variety of cardiovascular diseases, including angina pectoris, acute myocardial infarction and congestive heart failure. Current hypotheses of organic nitrate action propose that these compounds must first undergo metabolic activation (*via* denitration) to produce the active pharmacological species, NO, which then induces vascular smooth muscle relaxation (Ignarro *et al.*, 1981). Continuous administration of NTG has been shown to be associated with the development of pharmacological tolerance in patients either with chronic stable angina (Parker, 1987; Parker and Fung, 1984) or congestive heart failure (Jordan *et al.*, 1985). Although the mechanism of tolerance has not been completely delineated, it has been suggested that the impairment of the process of metabolic activation might be responsible (Needleman and Johnson, 1973; Marks *et al.*, 1989). The biochemical nature of this reaction, however, is not well understood.

GST have long been known to be an important enzyme system in the hepatic metabolism of organic nitrates (Keen *et al.*, 1976). Recently, GST isolated from human aorta also has been suggested to metabolize NTG (Tsuchida *et al.*, 1990). An inhibitor of GST, BSP, was reported to antagonize the *in vitro* vasodilating action of NTG (Yeates *et al.*, 1989). These results suggested that GST may be involved in the metabolic activation of NTG to NO in blood vessels. In these studies, however, the evidence for GST involvement in NTG metabolism was obtained primarily through formation of NTG metabolites (*e.g.*,

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ABBREVIATIONS: BSP, bromosulfophthalein; CDNB, 1-chloro-2,4-dinitrobenzene; GSH, reduced glutathione; GST, glutathione-S-transferase; NAC, N-acetyl-L-cysteine; NO, nitric oxide; NTG, nitroglycerin; RCD, redox chemiluminescence detector; SOD, superoxide dismutase; TSA, thiosalicylic acid; PMSF, phenylmethylsulfonylfluoride; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate; AUC, area under NO generation *vs.* time curve; GDN, glyceryldinitrate; Ve/Vo, elution to void volume ratio.

dinitrates and/or inorganic nitrite), with no corroborative demonstration that NO, the putative pharmacologically active species, was also produced. Thus, it is not clear whether the production of NO from organic nitrates in blood vessels utilize GST as the primary enzyme system.

Recently, we have developed a sensitive and specific analytical method for NO using redox chemiluminescence detection. We reported that NO production from NTG resided primarily in the microsomal suspension of the bovine coronary artery smooth muscle, and suggested that the enzymes might be bound to the plasma membrane (Chung and Fung, 1990). Although microsomal GST has been known to exist, the primary cellular GST activity is located in the cytosol. We, therefore, questioned whether microsomal GST is responsible primarily for NO generation from NTG in bovine coronary artery smooth muscle. Thus, we compared the activities of GST and NO generation from NTG in microsomes concerning their relative 1) temperature dependency, 2) cofactor (thiol) requirement and 3) behavior toward GST inhibitors. To test further whether the GST-mediated NTG metabolism leads to the production of NO, NO production from a purified liver GST preparation was compared to that of the microsomal suspension. In addition, we compared the functional molecular size of GST and the NO-generating system in microsomes by the radiation inactivation technique. Distinction between GST and NO-generating activity from solubilized microsomes was also demonstrated with gel filtration chromatography.

Methods

Materials. NTG aqueous solution (Perlinganit: 1.04 mg/ml in 5% glucose solution) was obtained from Pharma-Schwarz GmbH (Monheim, West Germany). GSH, SOD, purified liver GST, indomethacin, BSP, CDNB, PMSF and CHAPS were purchased from Sigma Chemical Co. (St. Louis, MO). Biogel A (1.5 m) and gel filtration standard were obtained from Bio-Rad Co. (Richmond, CA). All glassware used in this study was acid-washed and silanized to prevent NTG adsorption (Morrison and Fung, 1984).

Preparation of microsomes. Bovine hearts were obtained from a local slaughterhouse and transported to the laboratory while packed in ice. Both isolation of coronary arteries and preparation of microsomes from smooth muscle tissue have been described previously (Chung and Fung, 1990). The final protein concentration of the microsomal suspension was 1.0 to 1.5 mg/ml, as determined by the method of Lowry *et al.* (1951), using bovine serum albumin as a standard. The microsomal suspension was stored at −70°C and used within a week after preparation.

Quantitation of NO generation from NTG in microsomal incubation. Various initial concentrations of NTG (ranging from 0.091–1.466 mM) were incubated in the presence of adjuvants (*e.g.*, GSH, 13 μM and SOD, 100 U/ml) and 200 μg/ml of microsomal proteins in 50 mM phosphate buffer (pH 7.5). Addition of SOD in the incubation was necessary to stabilize the NO generated in solution (Chung and Fung, 1990). The incubation mixture was pre-equilibrated at 37°C for 15 min and the reaction was initiated by the addition of stock solutions of NTG. The total aqueous volume in the incubation was 2.5 ml. The incubation vials were sealed tightly with Teflon-faced rubber discs and stirred with Teflon-coated triangular-shaped magnetic stirrers. The headspace of the incubation vial was collected (100 μl) *via* a gas-tight syringe (Hamilton, Reno, NV) at 0, 30, 60, 90 and 120 min after NTG addition, and injected directly into a RCD (Model 207 B, Sievers Research, Boulder, CO) for the quantitation of NO. The extent of NO generation from NTG during the incubation was estimated by the AUC, corrected per 100 μl of headspace volume. The AUC, thus, had a unit of picomoles × minute per milligram of protein, and was numerically calculated by the linear trapezoidal method (Gibaldi and Perrier, 1982). The headspace AUC was used here because it is a convenient experimental parameter which we have shown previously to be directly proportional to the V_{max} of NO generation from NTG (Chung and Fung, 1990). The detector was calibrated by injecting various volumes of a gas mixture of 5.3 ppm NO in nitrogen (Linde Specialty Gases, Hamburg, NY). The detector response was linear ($r > 0.99$) over the concentration range tested (0.428–3.42 pmol of NO).

The kinetics of NO generation with various initial NTG concentrations were fitted to equation 1 using a nonlinear regression program in GraphPad (iSi Software, PA):

$$\frac{d[NO_{HS}]}{dt} = \frac{V_{max}[NTG]}{K_m + [NTG]} \quad (1)$$

where $[NO_{HS}]$ represented NO in the headspace of the incubation.

GST activity measurement. GST activity measurement in the microsomal suspension was carried out essentially according to Loscalzo and Freedman (1986). A mixture containing GSH (final concentration 1 mM) and the appropriate amount of protein was preincubated in the spectrophotometer (Cary model 108) at 25°C for 3 min. The following amounts of protein were used in each assay: for the microsomal suspension, 75 μg; for the purified bovine liver GST, 1.5 μg; and for fractions from the gel filtration chromatography study, 20 to 50 μg. CDNB (final concentration 1 mM) was added and mixed by inverting the cuvette to initiate the reaction, and the absorbance was recorded at 340 nm for 5 min. CDNB-GSH adduct formation was calculated from the slope of the absorbance change and its molar absorptivity (9.6 $mM^{-1}cm^{-1}$ at 25°C).

Effect of heat treatment on NO generation from NTG in microsomal incubation. To study the effect of heating on the production of NO, 1-ml aliquots of the microsomal suspension were heated at two different temperatures (*viz.*, 65°C and 90°C) for 1 hr. The final NTG concentration in the incubation mixture was 916 μM. NO generation in these heat-pretreated microsomes was monitored up to 120 min in the presence of GSH and SOD, as described above. GST activities in the heat-treated microsomal suspensions were also measured.

Effect of GST inhibitors on the NO generation. The effect of GST substrate (*viz.*, CDNB) and two inhibitors (*viz.*, BSP and indomethacin) on NO generation were examined separately, using incubation conditions similar to those described above. The final concentration of NTG in the incubation was 916 μM. The concentrations of GST substrate or inhibitors examined were as follows: CDNB, 400 μM; indomethacin, 0, 100, 400 and 800 μM; and BSP, 0, 100, 200, 400 and 800 μM. GST activities of the microsomal suspension in the presence of inhibitors were also determined.

Role of thiol in NO generation from NTG in microsomal incubation. To study the role of GSH in the NO generation from NTG, microsomes were incubated in the presence and absence of GSH (13 μM). In addition, the effects of GSH, TSA and NAC on NO generation were also examined. Both TSA and NAC were shown to promote NO production from NTG in human and rat plasma (Chong and Fung, 1990). The final concentration of GSH, TSA or NAC in the incubation was 1 mM, whereas the NTG concentration was 183 μM. The thiol was equilibrated in the incubation mixture for 15 min before NTG addition.

Measurement of inorganic nitrite, 1,2- and 1,3-GDN. To measure the metabolites of NTG (*i.e.*, inorganic nitrite, 1,2- and 1,3-GDN) simultaneously, 100-μl aliquots were taken from microsomal incubation or purified GST incubation at selected times. Ten μl of internal standard solution (isosorbide dinitrate, 20 μg/ml) and 25 μl of $CaCl_2$ (30 mg/ml) were then added. The mixture was vortexed for 10 sec, centrifuged for 1.5 min and 50 μl of the supernatant was directly injected into a high pressure liquid chromatography system equipped with an Econosphere C_8 column (3-μm particle size, Alltech Associates, Deerfield, IL). The flow rate was maintained at 0.7 ml/min using a mobile phase consisting of 18% methanol, 18% acetonitrile and 64%

phosphate buffer (50 mM, pH 2.3). The retention times of inorganic nitrite, 1,2-GDN, 1,3-GDN, isosorbide dinitrate and NTG were 5.1, 6.2, 5.6, 12.1 and 20.9 min, respectively. All peaks were base-line separated and the calibration curves of all compounds were linear ($r > 0.99$) in the concentration ranges studied.

Comparison of production of metabolites from NTG in incubations containing purified liver GST and microsome of bovine coronary artery smooth muscle cells. The purpose of this experiment was to compare the efficacy of NO production from the microsomes and a purified GST preparation, at the same degree of NTG metabolism, as judged by dinitrate production. Therefore, initial studies were conducted to determine the GST protein concentration that would yield comparable dinitrate formation to a reaction system containing 200 μg of microsomal protein/ml. A commercially available purified GST (Sigma, bovine liver GST; 8.5 U/mg solid, 1 U = 1.0 μmol of CDNB conjugated with GSH/min at 25°C at pH 6.5) was used to prepare incubation mixtures containing 4, 4.8, 6, 7.2 and 8 μg of purified GST in 1 ml. At 1 hr after the addition of NTG, 100-μl samples of these incubations were analyzed for total dinitrate formation. It was determined that an incubation mixture containing 6.8 μg/ml of purified GST yielded the same total dinitrate amount (3.6 μM) as that produced from microsomal incubation.

In a subsequent experiment, production of dinitrate metabolites, nitrite ion and NO were monitored in two incubation mixtures, one utilizing 6.8 μg/ml of purified liver GST and another utilizing the microsomal suspension (200 μg protein/ml). In addition, the incubations contained NTG (916 μM), GSH (13 μM) and SOD (100 U/ml). One hr after the introduction of NTG, 100 μl of headspace was taken and analyzed for NO production. In addition, 100 μl of the aqueous mixture was collected for high pressure liquid chromatography assay for inorganic nitrite and dinitrate metabolites.

Controlled experiments were also performed to examine the distribution behavior of NO to headspace from the incubations with purified liver GST and the microsomes. The compositions of the incubation mixtures were identical to those described above, except that the NTG solution was replaced by its vehicle (5% dextrose). The stock NO solution, prepared by carefully diluting a saturated NO solution (Chung and Fung, 1990), was added to the aqueous phase in a tightly sealed vial to yield an anticipated NO concentration of 5 pmol/100 μl headspace. Headspace NO was analyzed at 15, 30, 45 and 60 min after the addition of NO.

Radiation inactivation. One-ml aliquots of the microsomal suspension were plated onto 1.2-mm deep aluminum trays and frozen immediately by immersion in liquid nitrogen. The frozen microsomal suspension sample trays were placed in a closed chamber and irradiated with 1.5 MeV electrons generated from a Van de Graaff accelerator. The chamber temperature was maintained at −45° to −50°C with liquid nitrogen vapor. The radiation dose received by each sample tray was controlled by varying the number of passages of the electron beam across the sample. The radiation dose was quantified at the sample inactivation temperature using Blue Cellophane film (Du Pont MSC-300) calibrated against a Fricke dosimeter (Jung *et al.*, 1980). The total radiation dose ranged from 0 to 14 Mrad. The irradiated microsomal suspension were stored at −70°C until they were analyzed for both GST activity and NO generation using NTG as a substrate.

Target size analysis. The radiation inactivation data were analyzed according to a "single-target, single-hit" model of radiation inactivation (Kempner and Schlegel, 1979; Jung, 1984). Because the survival activity declined exponentially with respect to radiation dose, the slopes were determined by the method of least squares linear regression and the target sizes were calculated according to equation 2.

$$Mr = 6.4 \times 10^5 / D_{37} \quad (2)$$

where D_{37} is the radiation dose (in Mrad) required to induce 37% residual activity, and Mr is the molecular size of the target in daltons.

Solubilization of microsomes from bovine coronary artery smooth muscle cells. Microsomes were obtained using approximately 20 g of bovine coronary artery smooth muscle tissue by the procedures described above. The microsomes were suspended in 1.5 ml of 250 mM sucrose containing 1 mM PMSF and 3% CHAPS. The samples were stirred at 4°C for 1 hr and centrifuged at 100,000 × *g* for 1 hr. The supernatant was collected and used in the subsequent experiment. Under these conditions, both NO-generating activity from NTG and GST activity were solubilized from the membrane.

Gel filtration chromatography. The detergent-solubilized extract containing 4 mg of protein was loaded on a Biogel A 1.5-m column (1.5 × 75 cm) pre-equilibrated with a buffer (10 mM phosphate, 0.5% CHAPS and 1 mM PMSF, pH 6.8). Fractions were collected at 4 ml each, and analyzed for NO-generating activity from NTG and GST activity as described earlier. NO generation was measured after 4 hr of incubation of the fraction with NTG and cofactors.

Statistical analysis. $P < .05$ was accepted as denoting statistical significance. Data are expressed as mean ± S.D.

Results

The profile of NO generation rate *vs.* NTG concentration exhibited classical Michaelis-Menten kinetics (fig. 1). This observation is consistent with the view that the production of NO from NTG is enzymatic in origin. The estimated kinetic parameters obtained from fitting the data in figure 1 to equation 1 were: K_m = 285 μM and V_{max} = 3.89 pmol of NO in 100 μl headspace/mg protein/hr. These apparent Michaelis-Menten parameters, however, were obtained using headspace NO concentrations, and they, therefore, do not represent the true K_m and V_{max} values for the reaction in solution.

Production of NO from NTG apparently increased by 2.5-fold in microsomes which were pretreated at 65°C. Controlled studies which examined the effect of heating on the partitioning of NO in microsomes indicated that both pretreatment conditions (at 65 and 90°C) increased NO partitioning into headspace by only 1.3-fold (E.A. Kowaluk and H.-L. Fung, unpublished data). These results suggested, therefore, that the NO-generating enzyme system can be activated by heating at 65°C (fig. 2). In comparison, pretreatment of microsomes at 90°C for 1 hr did not significantly alter the apparent NO headspace concentration compare to control (*i.e.*, unheated microsomes). These observations suggested that there may be an optimal temperature for heat activation of the NO-generating activity from NTG in microsomes. In contrast, GST activity was significantly impaired by heating, both at 65° and 90°C for 1 hr (fig. 2). Thus, the heat lability profiles of NO generation and GST activity in microsomes were apparently different.

Addition of CDNB in the incubation significantly reduced NO generation from NTG (table 1, $P < .001$). Because CDNB also utilizes GSH as a cofactor, it is possible that CDNB may inhibit NO production from NTG by consuming GSH. This interpretation can be ruled out by the observation that addition of GSH regenerating system in the incubation did not restore the NO-generating activity of microsomes (table 1, $P < .001$). NO-generating activity was not critically dependent on GSH presence, because GSH absence only reduced the activity by about 44% (table 1). In contrast, GST activity was reduced to only about 5% of control in the absence of GSH (*i.e.*, GST activity with GSH = 12.2 ± 0.590 nmol/mg/min; GST activity without GSH = 0.597 ± 0.277 nmol/mg/min, $P < .001$).

NO generation from NTG was also catalyzed by thiols other than GSH. Among three thiols examined, NAC was most active in catalyzing NO generation, whereas GSH was the least active. The extent of NO generation, as estimated by the AUC of headspace NO (in the unit of pmol per 100 μl headspace ×

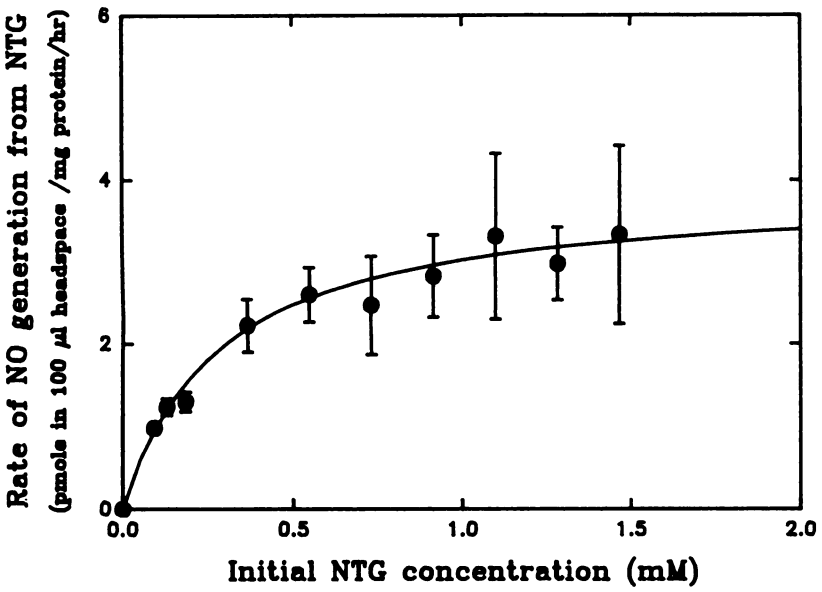

Fig. 1. NO generation at various initial concentrations of NTG in incubations of microsomes of the bovine coronary artery smooth muscle cells. Data are expressed as means ± S.D. (n = 3–8 at each NTG concentration).

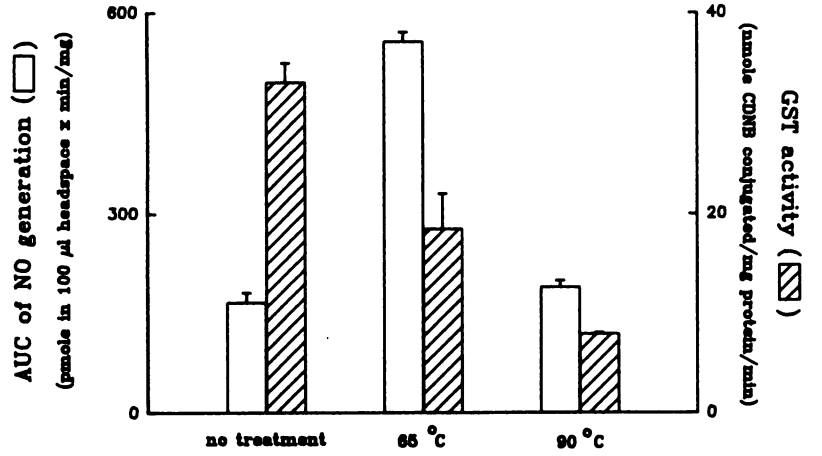

Fig. 2. The effect of heat pretreatment on GST and NO-generating activities. Data are expressed as means ± S.D. (n = 3).

TABLE 1

Inhibition of NO generation from NTG by CDNB in microsomes of the bovine coronary artery smooth muscle cells

Cofactor Added	n Runs	AUC of NO Generation	
		Without CDNB	With CDNB
		pmol NO/100 µl headpsace × min/mg protein	
GSH (4 µg/ml)	6	239 ± 17.1	28.7 ± 0.333***
GSH renegerating system[a]	6	225 ± 14.4	38.9 ± 2.87***
GSH regenerating system × 2	3	275 ± 24.4	76.7 ± 3.14***
No GSH	3	134 ± 16.6	

[a] Adopted from Fung *et al.* (1984).
*** Different to the corresponding control (*i.e.*, without CDNB), P < .001.

min/mg protein), were 226 ± 11.2 for GSH, 350 ± 33.7 for TSA and 397 ± 5.79 for NAC, respectively.

Both GST inhibitors (indomethacin and BSP) decreased apparent GST activity toward CDNB in a concentration-dependent manner (fig. 3), but they appeared to have little or no effect on NO generation from NTG in microsomes.

Figure 4 consolidates all the results of the comparative studies involving GST activity and NO generation that have been described so far, using data obtained from manipulations such as heat pretreatment, and the addition of GST inhibitors and thiol cofactors. It is clear that NO-generating activity and GST activity in microsomes do not covary. The slope of the linearly regressed line (dotted line) was not statistically different from zero (n = 19, r = 0.21).

Table 2 shows the extents of metabolite production when NTG was incubated in purified liver GST *vs.* microsomes of the bovine coronary artery smooth muscle. As planned, the dinitrate production was similar in both experiments. Importantly, NO production was about 2.5 times higher from the microsomal incubation, whereas GST activity (as expected) was 3.5 times higher from the purified liver GST incubation. The apparent differences in NO headspace concentration were not due to an alteration in NO distribution into headspace, because, in the controlled experiment, we found the extent of NO partitioning into headspace not to be significantly different in these two incubation systems (AUC for purified liver GST and microsomal incubations were 110 ± 9.5 and 110 ± 5.2 pmol × min, respectively). Thus, when NO production was corrected per unit of GST activity, the microsomal preparation was estimated to be about 8 times more efficient in generating NO than purified liver GST. Interestingly, inorganic nitrite ion production from both preparations was similar, indicating that nitrite ion is not a reliable index of NO production from NTG.

Increasing radiation exposure of the microsomal suspension caused inactivation of both NO-generating and GST activities

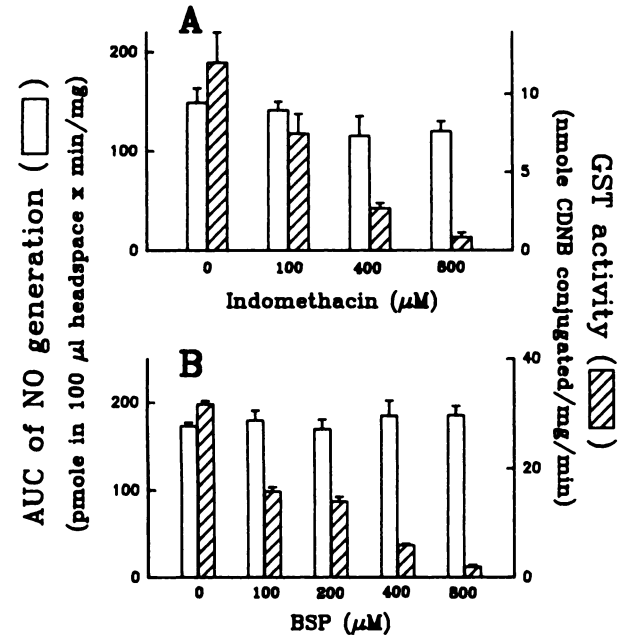

Fig. 3. The effect of GST inhibitors on NO-generating activity from NTG and GST activity in microsomes. Panels A and B represent the effects of indomethacin and BSP, respectively. Data are expressed as mean ± S.D. (n = 3 at each inhibitor concentration).

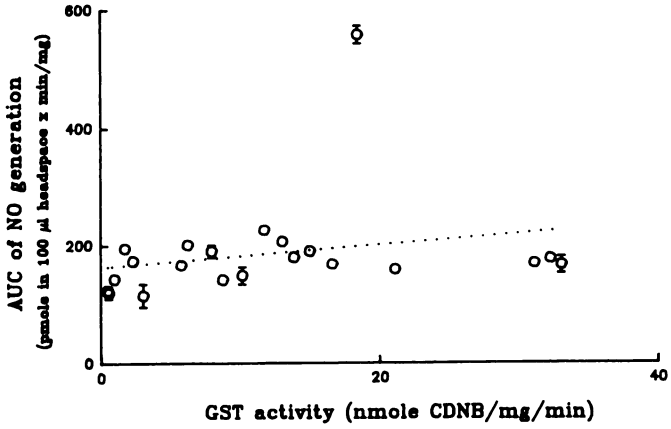

Fig. 4. Correlation between GST activity and NO generation from NTG in microsomes under different conditions of heat treatment, thiol addition and inhibitor presence. (r = 0.21, n = 19, P > .05).

TABLE 2

Comparison of the activities of the bovine coronary artery smooth muscle cell microsomes and purified bovine GST toward NTG and CDNB

	Microsomes	Purified Bovine Liver GST
Total dinitrate (nmol/ml)	7.76 ± 0.15	8.04 ± 0.54
NO in headspace[a]	2.47 ± 0.19	1.01 ± 0.090***
Inorganic nitrite (nmol/ml)	9.33 ± 1.76	9.23 ± 0.23
GST activity[b]	0.0401 ± 0.0027	0.145 ± 0.006***
NO in headspace/GST activity	61.7 ± 4.5	8.08 ± 0.40***

[a] pmol in 100 μl headspace at 1 hr after the start of incubation.
[b] nmol CDNB conjugated/2.5 ml, using the identical protein concentrations as those in the NTG experiment.
*** Different from incubation with microsomes, P < .001.

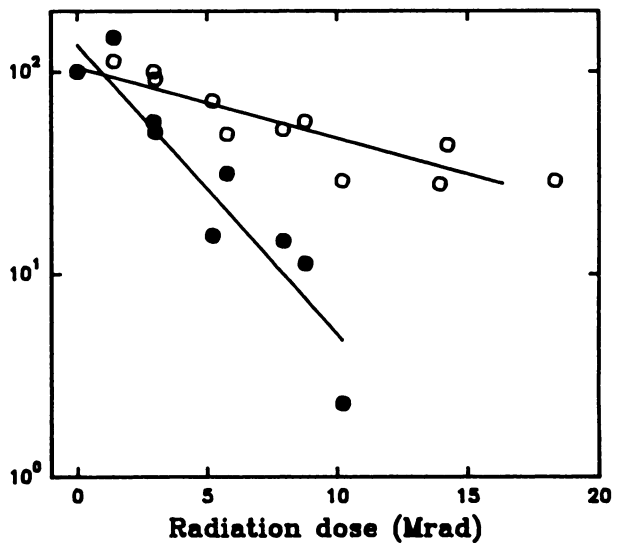

Fig. 5. Radiation inactivation of GST and NO-generating activity in microsomes. The data were obtained from triplicate experiments involving two different batches of microsomes. The solid lines represent linear regression lines for GST activity (O) and NO-generating activity (●).

(fig. 5). The semi-logarithmic plots of activity *vs.* radiation dose for both biochemical reactions were linear over the range used (*i.e.*, 0–14 Mrad). Based on the D_{37} values calculated from the respective slopes, the apparent molecular weights, expressed as means (95% confidence intervals) of the NO-generating system in microsomes and microsomal GST were estimated to be 160 (128–210) and 54 (46–67) kD, respectively.

NO-generating activity was clearly dissociated from GST activity when solubilized microsomes were subjected to gel filtration (fig. 6). The peak activity of NO generation was observed at the position corresponding to Ve/Vo of 1.7, whereas peak GST activity was measured at 2.2 Ve/Vo (fig. 6A). Protein elution, determined by the optical density at 280 nm, was still present up to 2.5 Ve/Vo. Molecular weights of the two enzymes determined from standard protein elution (fig. 6B) were 49 kD for GST activity and 210 kD for NO-generating activity, respectively.

Discussion

Both the action and inaction of organic nitrates appear to involve the biochemical conversion of the nitrate group to NO. Vasodilatation is brought about by successful conversion, whereas pharmacological tolerance is believed to come about when this conversion process is diminished or interrupted. The classical hypothesis of nitrate tolerance suggests that critical sulfhydryl groups necessary as cofactors for this reaction are depleted during sustained nitrate exposure (Needleman and Johnson, 1973), thereby leading to diminished response when new doses of nitrates are administered (Marks *et al.*, 1989; Parker *et al.*, 1984; Bauer and Fung, 1990).

Currently, no suitable clinical strategy exists which can avoid the development of this tolerance phenomenon while maintaining continuous nitrate administration. It would seem logical that any rational attempts to solve this important therapeutic problem would require a thorough understanding of the nature of the critical metabolic activation process to produce NO, and the factors which affect the extent and rate of production. However, little is known about this reaction, including the

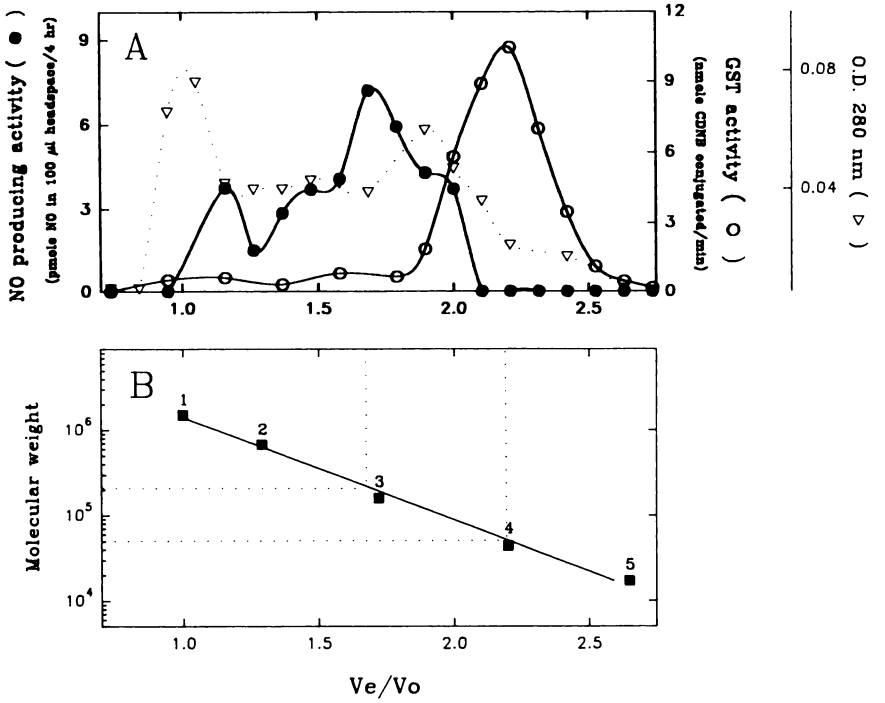

Fig. 6. Gel filtration chromatography of solubilized microsome. Panel A shows the activity profile of NO generation from NTG (●) and GST (O) *vs.* Ve/Vo. The dotted line denotes optical density (O.D.) at 280 nm (▽). Panel B shows the elution of standard proteins on the gel filtration column. The molecular weights of peak NO-generating activity and GST activity could be estimated from this plot by interpolation. The number above each point represents the standard protein utilized for molecular weight determination (1: blue dextran, m.w. > 1.5×10^6; 2: bovine thyroglobin, m.w. = 670,000; 3: bovine γ-globin, m.w. = 158,000; 4: chicken ovalbumin, m.w. = 44,000; 5: horse myoblobin, m.w. = 17,000).

identity of the enzyme system(s) that is (are) involved in this process.

We have shown previously that NO indeed can be produced from bovine coronary smooth muscle tissue *in vitro* when NTG is added as a substrate. We further showed that the conversion system appeared to be located in the microsomes and was bound to the plasma membrane fraction. We now show that NO-generation from NTG in microsomes is a saturable process (fig. 1), which is consistent with the view that the biochemical activation of NTG to NO is mediated enzymatically. Blank incubations, which contained every ingredient except the microsomes, were run, and nonenzymatic production of NO was found to account for less than 20% of total NO production. This result is consistent with the finding of Feelisch and Noack (1987), who showed that spontaneous NO production with 5 mM GSH was not detectable in their system. It is recognized that the concentration of NTG used (0.092–1.5 mM) was considerably higher than those that will typically elicit relaxation (1 nM to 10 μM). It is not clear whether this discrepancy is due to the experimental system itself (*i.e.*, subcellular fraction *vs.* intact vascular tissue), or the lack of optimal conditions for NO production (notably cosubstrate concentrations) or for NO detection (*e.g.*, SOD concentration).

The apparent inhibition of NO generation by the GST substrate, CDNB, may suggest that conversion of NO from NTG may indeed be catalyzed by GST. This inhibition could also arise from cofactor (GSH) depletion rather than direct competition, because GST utilizes GSH in the conjugation reaction with CDNB. However, NO generation remained inhibited by CDNB in the presence of a GSH-regenerating system. Inhibition of NO generation by CDNB, therefore, is likely to be due to mechanisms other than GSH depletion.

Other evidence in this study, however, suggested that GST was not primarily involved in the conversion of NTG to NO. GST activity was not altered in parallel to that of NO generation when several manipulations were performed (*e.g.*, heat pretreatment, GST inhibitor addition and thiol requirement; fig. 4). GST activity could be varied over 200-fold through these manipulations, but NO generation was relatively unaffected (a range of only 2.5-fold) by the same probes.

The exact mechanism for the apparent enhancement of NO production after heat pretreatment is not known. Changes in the nonprotein component of the membrane (*e.g.*, increased lipid oxidation from heating) may be possible, but the role of lipid-supported catalytic reduction is probably unimportant, because detergent extracts of microsomes, when applied to gel filtration chromatography, showed the activity to reside at about 210 kD, a molecular size too large for lipids. Furthermore, on-going (and unpublished) studies in our laboratory show that the NO-generating activity can be retained on an ion exchange column and eluted by a salt gradient, a property which is more consistent with catalysis by proteins than by lipids.

Because CDNB is one of the more sensitive substrates toward many isozyme forms of GST (Keen *et al.*, 1976), it is expected that inhibitor concentrations that can decrease GST activity would also diminish NO generation if both reactions are mediated by the same enzyme. NO generation was also not critically dependent on GSH as the sole thiol cofactor because NAC and TSA both can catalyze the reaction. Although the feasibility of NAC or TSA as a cofactor for GST activity in our preparation was not examined directly, Keen *et al.* (1976) have reported that the metabolism of organic nitrates by GST specifically required GSH, which could not be replaced by other thiols, including NAC and L-cysteine. Thus, if GST is the enzyme which catalyzes the conversion of NTG to NO, then both TSA or NAC would not be expected to enhance NO production in microsomes.

Another evidence of the dissimilarity between GST and the predominant enzyme that converts NTG to NO in microsome was obtained through a purified bovine liver GST preparation. We compared the metabolite profiles of NTG when it was incubated in microsomes *vs.* this standard GST preparation. At the same level of production of total dinitrates, the principal metabolites of NTG, both NO production and GST activity

were shown to be different. When corrected for GST activity, microsomes of the bovine coronary artery smooth muscle were shown to be about 8 times more active in producing NO than purified liver GST.

These results suggest that the NO-generating enzyme in microsomes is not identical to the purified bovine liver GST. Other isozymes of GST, of course, exist, and it is possible that the microsomal enzyme responsible for NO generation is an isozyme of GST distinct to that found in bovine liver. If this were the case, however, then this bovine coronary enzyme is quite different in its relative activity toward CDNB and NTG than purified bovine liver GST.

Radiation inactivation target-size analysis also revealed that the NO-generating system is different from GST, based on a large difference observed in the functional molecular sizes (160 *vs.* 54 kD) of these two activities. Consistent with radiation inactivation study, the gel filtration chromatography experiment revealed molecular weights of 210 kD for NO generation and 49 kD for GST, both within the 95% confidence intervals of the corresponding molecular weights estimated from the radiation inactivation study. Because of the small amounts of protein found in each of the fraction, the incubation time had to be extended to 4 hr for sufficient NO to be produced. NO generation activity was not manifested in a single peak from this gel filtration column, in part possibly due to the formation of aggregates and fragments after solubilization and manipulation. However, the chromatographic data clearly showed that the NO-generating activity was distinctly dissociated from GST activity in the microsomal preparation.

Tsuchida *et al.* (1990) reported that several GST isozymes isolated from human aorta exist as homo- or heterodimers, and the molecular size of each subunit was 27 kD. This observation is consistent with our finding regarding microsomal GST, although a direct comparison might not be feasible because there are differences in the preparations (*e.g.,* human *vs.* bovine, soluble GST *vs.* microsomal GST).

An interesting observation from this study is that inorganic nitrite ion production was not parallel to that of NO when NTG was used as the substrate. Some studies have utilized nitrite production as an index of vascular metabolism of organic nitrates (Ignarro *et al.,* 1981; Axelsson and Karlsson, 1984). Our results suggest, however, that nitrite ion production may stem from more than one reaction source, and it did not reflect the difference in the extent of production of the pharmacologically pertinent intermediate (*viz.,* NO). Ignarro *et al.* (1981) have suggested that organic nitrates such as NTG are first converted to nitrite ion, which in turn produces NO. This reaction sequence has been challenged by Romanin and Kukovetz (1988), who showed that nitrite ion formation from organic nitrates cannot account for the extent of guanylate cyclase activation observed. Our data also suggested that nitrite ion is probably not an obligatory intermediate for the formation of NO in microsomes.

The use of nitrite ion as an index of NO production has now been extended to work relating to the conversion of the endogenous substrate L-arginine to NO in, for example, the vascular endothelium, macrophage and the nervous system (Collier and Vallance, 1989). A recent publication (Tracey *et al.,* 1990) also suggested that a diazotization reaction for NO (which measured inorganic nitrite) was not correlative with biological assays for NO and endothelium derived relaxing factor.

In conclusion, we have shown, by biochemical, biophysical and chromatographic characteristics, that the conversion of NTG to NO in microsomes of the bovine coronary artery smooth muscle is not primarily mediated by GST. Because pharmacological tolerance toward NTG and other organic nitrates during chronic therapy is thought to be possibly derived from a dysfunctioning of the metabolic activation process, it would appear necessary to gain a more thorough understanding of this biochemical reaction, including the enzyme systems involved. Purification and identification of the nitrate-NO converting enzyme in microsomes from the bovine coronary artery smooth muscle cells are currently being carried out in our laboratory.

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