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RELATIONSHIP BETWEEN NITROGLYCERIN-INDUCED VASCULAR RELAXATION AND NITRIC OXIDE PRODUCTION

PROBES WITH INHIBITORS AND TOLERANCE DEVELOPMENT

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Abstract—We have shown previously that nitric oxide (NO) is generated from nitroglycerin (NTG) through enzyme-mediated reactions in the bovine coronary artery smooth muscle cell, but it is not known whether this metabolic conversion plays a significant role in the pharmacologic action of NTG, viz. relaxation. In this study, we developed a technique that allowed direct measurement of NO from intact bovine coronary arterial rings that were incubated previously with NTG, and examined whether changes in NTG-induced relaxation were accompanied by parallel changes in NO generation. Co-incubation of the vascular preparations with a potent inhibitor of glutathione-*S*-transferases (GSTs), bromosulfophthalein (up to 200 μM), did not affect NTG-induced relaxation, nor did it alter NO generation from NTG in the preparation. In contrast, 1-chloro-2,4-dinitrobenzene (CDNB), a GST substrate, inhibited NO generation as well as the relaxation response of NTG in the intact vascular tissue preparation. CDNB, however, did not decrease the relaxant responses of nifedipine and isoproterenol. Thus, the inhibitory effect of CDNB on NTG-induced relaxation and NO production appeared specific. When bovine coronary rings were made tolerant to NTG by pretreatment with 0.44 mM NTG for 1 hr, the EC_{50} was shifted to the right 162-fold, and NO generation was also reduced in intact rings and tissue homogenates. However, when the homogenates were further subfractionated to microsomes and cytosols, or when homogenates were allowed to stand for a similar time period necessary for subfractionation, the difference in NO production from control versus tolerant tissue preparations disappeared. It is possible, therefore, that the NTG-induced tolerance process might have been partially reversed during this time period. Results of this study identified CDNB as an apparently specific inhibitor of NTG action, but showed that GST-mediated reactions were probably not involved in the metabolic activation of NTG. Our results also indicated that tissue NO generation from NTG was positively related to the relaxation responses generated by this nitrovasodilator.

It is generally believed that the vasodilating activity of organic nitrates, including nitroglycerin (NTG)†, arises as a result of their vascular metabolism to nitric oxide (NO) in vascular smooth muscle cells [1]. The NO produced then activates soluble guanylate cyclase, enhancing the production of cGMP, thus causing vasodilation [1]. Development of nitrate tolerance is also believed to be accompanied by reduced metabolism of nitrate in vascular smooth muscle cells [2]. The enzyme responsible for the converting process, however, is not yet completely defined. Recently, we showed that NO generation in subfractions of bovine coronary artery smooth muscle cells is well correlated with the activities of two marker enzymes of plasma membrane, thus suggesting that the NTG–NO converting enzyme may be membrane-bound [3]. Subsequently, we showed that the conversion activity is not identical to membrane-bound glutathione-*S*-transferase (GST) [4], an enzyme previously believed to be responsible for the metabolism of NTG [5, 6].

Although the membrane-bound NTG–NO converting enzyme has been characterized partially (Seth P and Fung H-L, unpublished observations), its role in contributing to the *in vitro* NTG relaxation is not fully understood, nor is the general relationship between relaxation and metabolic activation of NTG to NO. While several studies have suggested that metabolic conversion to the dinitrate metabolites is linked to the vasorelaxant effects of NTG [2], no study has explored the relationship between the ability of vessel segments to generate NO and their ability to dilate. To test this relationship, it becomes necessary to develop the capability of monitoring NO generation from NTG in intact vascular preparations. This was one of the objectives of this study. After this technique was established, we examined the effects of two potential inhibitors on NTG-induced relaxation and NO generation in intact vascular tissues. The two inhibitors chosen, bromosulfophthalein (BSP) and 1-chloro-2,4-dinitrobenzene (CDNB), were selected based on our previous experience with these compounds when

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† Abbreviations: NTG, nitroglycerin; NO, nitric oxide; GST, glutathione-*S*-transferase; BSP, bromosulfophthalein; CDNB, 1-chloro-2,4-dinitrobenzene; PMSF, phenylmethylsulfonyl fluoride; RCD, redox chemiluminescence detector; and SOD, superoxide dismutase.

they were used to probe the NO-generating ability from NTG in microsomes of the bovine coronary artery smooth muscle cell [4]. In addition, we also examined the effects of tolerance development on NO production in intact vascular segments.

MATERIALS AND METHODS

Materials. NTG aqueous solution (Perlinganit®, 1.04 mg/mL in 5% dextrose solution) was obtained from Pharma-Schwartz GmbH (Monheim, West Germany) and, when necessary, was diluted with 5% dextrose solution. BSP, CDNB, phenylmethylsulfonyl fluoride (PMSF) and superoxide dismutase (SOD) were purchased from the Sigma Chemical Co. (St. Louis, MO). All glassware used in this study was acid-washed and silanized to prevent drug adsorption [7].

Isolation of bovine coronary artery for vasorelaxation study. Capped bovine hearts were obtained from a local slaughterhouse. Hearts were isolated from animals and transported (within 40 min) packed in ice, to the laboratory. The left circumflex coronary arteries were isolated and immediately immersed in ice-cold Kreb's buffer, which was saturated previously with 5% CO_2 in O_2. The composition of Krebs buffer [8] was (in mM): NaCl, 120; KCl, 5.6; $MgCl_2$, 1.2; NaH_2PO_4, 1.2; dextrose, 10; $NaHCO_3$, 25; and $CaCl_2$, 2.5. After isolation, excess fat and connective tissues were carefully cleaned from the arteries, which were then cut into rings (2–3 mm in thickness and 4–6 mm in diameter) using surgical scissors. Typically, each coronary artery of approximately 20 cm in length could yield at least 15–20 segments for study.

Vasorelaxation study [9, 10]. Each ring was mounted using fine stainless steel wires in a 10-mL jacketed tissue bath containing Kreb's buffer maintained at 37° and gassed constantly with 5% CO_2 in O_2. Ring segment tension was measured isometrically using a force-displacement transducer (FT03C, Grass Instruments, Quincy, MA) and was recorded on a Grass model 79D Polygraph. The rings were subjected to an optimal resting tension of 4 g. Ring segments were washed every 20 min during the initial equilibrium period with fresh Kreb's buffer, and were progressively stretched until constant tension was achieved. The rings were then allowed to equilibrate further for 2 hr, with fresh buffer washes every 20 min. After equilibration, the ring preparations were exposed to 120 mM K^+ Kreb's buffer (in which NaCl has been replaced with KCl). After the contractile response reached its maximum, the ring segment was allowed to return to base-line tension by a buffer wash. Submaximal tones were then induced by adding 30 mM K^+ Kreb's buffer, and relaxation studies were carried out.

Treatment of the bovine coronary ring preparation with inhibitors. In the BSP studies, arterial ring preparations were contracted as described, using K^+ (for 10 min). BSP solutions (100 μL), dissolved in Kreb's buffer, were added to achieve final concentrations of 0, 100 and 200 μM. Fifteen minutes after BSP addition, a cumulative concentration–response curve to NTG was determined.

In the CDNB studies, NTG-induced relaxation was determined at a fixed NTG concentration (200 nM), but with various concentrations of CDNB. Relaxation responses to NTG in each ring preparation were determined three times, first without CDNB (control), then with CDNB, and again without CDNB. In each relaxation segment, rings were contracted with 30 mM K^+ Kreb's buffer and 10 μL of CDNB or ethanol (control vehicle) was added to the tissue bath. Fifteen minutes later, NTG was added and the relaxation responses were recorded. The ring segments were then washed with Kreb's buffer and equilibrated for 15 min, and the procedure was repeated again.

In a separate experiment, the effect of CDNB on the relaxation responses of two non-nitrate vasodilators, viz. isoproterenol and nifedipine, was examined. Preliminary studies were performed to determine the appropriate drug concentrations that would achieve a degree of relaxation similar to that observed with 200 nM NTG. These concentrations were 1.2×10^{-7} M for isoproterenol and 2×10^{-7} M for nifedipine. In these experiments, the ring preparations were mounted, equilibrated and contracted as described above. Fifteen minutes after the addition of control vehicle (10 μL ethanol), isoproterenol or nifedipine at the specified concentrations, vascular relaxation reponses were recorded. The rings were then washed with fresh buffer, allowed to equilibrate for 15 min and re-contracted, and 10 μL of ethanolic CDNB solution (final concentration 160 μM) was added. After 15 min, vasodilator-induced relaxations were again determined.

Induction of in vitro *nitrate tolerance in bovine coronary artery rings.* For the development of NTG tolerance, vascular rings were incubated with 0.44 mM NTG for 1 hr. Control ring segments were treated identically with 5% dextrose solution. After the pretreatment period, ring segments were washed six times every 5 min with fresh buffer [8]. Then, the rings were submaximally contracted with 30 mM K^+ Kreb's buffer as described earlier and the cumulative concentration–response curves to NTG were determined.

NO *measurement from the bovine coronary artery ring preparation.* Bovine coronary arterial rings (approximately 200 mg) were blotted, weighed, and added to a micro-reaction vial (6.34 mL, Alltech Associates, Inc., Deerfield, IL). Kreb's buffer containing 30 mM K^+ was added to adjust the total volume to 2.3 mL, along with 50 μL of SOD solution (final concentration 100 U/mL). When necessary, either ethanolic CDNB solution or BSP solution (in Kreb's buffer) was added to the incubation mixture. The final concentrations were 160 μM for CDNB and 200 μM for BSP. The incubation mixture was preincubated for 15 min with stirring. At time zero, 200 μL of NTG aqueous solution (final concentration 366 μM) was added and the reaction vial was sealed from ambient air. Aliquots (100 μL) of headspace samples were taken via a gas-tight syringe (Hamilton, Reno, NV) at 15, 30, 45 and 60 min after substrate introduction and injected into a redox chemiluminescence detector (RCD; model 207 B, Sievers Research, Boulder, CO) [3]. The detector condition and calibration procedure were described

previously [3]. Measurement of NO from tolerant rings was carried out in the same manner, using six ring segments at a time. The area under the NO generation versus time curve (AUC) was calculated based on the linear trapezoidal rule [11] and used to express the activity of NO production [3].

Controlled experiments were also performed to examine the distribution behavior of NO to headspace in control and tolerant ring incubations. The composition of the incubation mixtures was identical to those described above except that the NTG solution was replaced by its vehicle (5% dextrose). A stock NO solution, prepared by diluting a saturated NO solution [3], 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 NO addition.

NO *generation from microsomes of bovine coronary artery smooth muscle cells.* Microsomes were prepared from bovine coronary artery smooth muscle cells, as described previously [4]. Cofactors (GSH, 13 μM and SOD, 100 U/mL) were added to aliquots of microsomes at a protein concentration of 200 μg/mL in pH 7.5, 50 mM phosphate buffer. CDNB (160 μM) or BSP (200 μM) was added to the microsomal preparation and incubated for 15 min with stirring. The reaction was initiated by adding 200 μL of aqueous NTG solution and the reaction vial was sealed from ambient air. The final NTG concentration was 366 μM. Aliquots of headspace (100 μL) were collected via a gas-tight syringe at 30, 60, 90 and 120 min, and analyzed via the RCD. The AUCs in the presence and absence of inhibitors were calculated and compared.

Preparation of subfractions from NTG-tolerant ring preparations. Bovine coronary artery ring segments were pretreated with NTG solution or NTG vehicle as described earlier. After washing with fresh buffer, approximately 500 mg of tissue was placed in a round-bottomed plastic tube and 5 mL of 250 mM sucrose with 500 μM PMSF solution was added. After mincing with scissors, the tissue was homogenized with a Polytron (PT 10/35 Brinkmann Instruments; Westbury, NY) fitted with a PTA 7 generator. The homogenate was filtered through two layers of surgical gauze and collected for study. When necessary, the homogenate was incubated at 4° for 2 hr and NO-generating activity was subsequently determined. In one study, homogenates from control and NTG pretreated tissues were further subfractionated to corresponding microsomes and cytosols, as described previously [4]. The microsomal fraction was suspended in 2 mL of a solution containing 250 mM sucrose and 700 mM KCl. The protein concentrations in subfractions were determined by the method of Lowry *et al.* [12], using bovine serum albumin as a standard.

NO *generation from NTG in cellular subfractions.* The relevant subfraction was added to an incubation vial along with SOD (100 U/mL final concentration). The volume was adjusted to 2.3 mL by 50 mM phosphate buffer (pH 7.5). The mixture was preincubated for 15 min while stirring and, at time zero, 200 μL of NTG solution (final concentration 366 μM) was added. Headspace samples (100 μL) were taken at 30, 60, 90 and 120 min after introduction of the substrate, and the NO generated was measured.

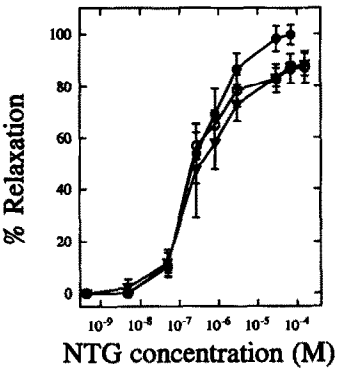

Fig. 1. Effect of BSP on NTG-induced vascular relaxation. Data are means ± SD. Key: control (○, N = 5); in the presence of 100 μM BSP (●, N = 6); and in the presence of 200 μM BSP (▽, N = 4).

Data analysis. The NTG concentration versus relaxation curves were fitted to the Hill equation (Eqn 1), using nonlinear regression with the computer program GraphPad (iSi Software, PA):

$$\text{Effect} = \frac{E_{max} \cdot [\text{NTG}]^s}{EC_{50} + [\text{NTG}]^s} \quad (1)$$

where E_{max} is the maximum vasodilating effect, EC_{50} is the NTG concentration which produces a half-maximal effect, and s represents the Hill's slope. $P < 0.05$ was accepted as denoting statistical significance. Data are expressed as means ± SD.

RESULTS

Within the concentration range tested, BSP treatment did not produce any significant effect on NTG-induced relaxation (Fig. 1). Based on the parameter estimation by non-linear regression using Hill's equation, the EC_{50} values for BSP treatment groups were not different from that of the control (EC_{50} values were 175 ± 37, 226 ± 59 and 233 ± 42 nM for control, 100 μM BSP and 200 μM BSP, respectively). The E_{max} and s (slope) values for BSP pretreatment groups obtained from computer fitting were also not different from those of the control. BSP addition did not affect the contraction produced by 30 mM K^+ Kreb's buffer (data not shown).

In contrast, CDNB inhibited NTG-induced relaxation in a concentration-dependent manner (Fig. 2), although it did not have any apparent effect on the contraction induced by K^+ (data not shown). The inhibitory effect of CDNB did not appear to be rapidly reversible, since washing with buffer followed by 15 min of equilibration did not reverse the CDNB effect (Fig. 2). In fact, from 2 to 80 μM CDNB, the inhibitory effect after the buffer wash appeared even more pronounced, suggesting that the CDNB effect may be delayed.

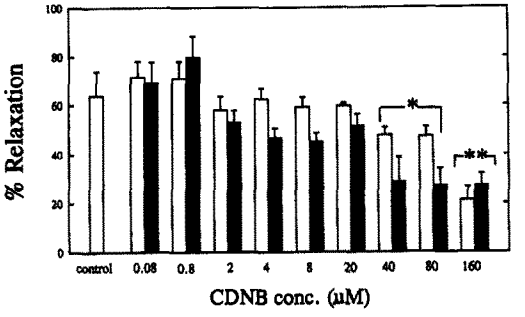

Fig. 2. Effect of CDNB on the relaxation response toward 200 nM NTG. Data are means ± SD. Key: in the presence of CDNB (□, N = 3–4) and after CDNB was washed out and the ring preparation was re-challenged with NTG (■, N = 3–4); (*) denotes $P < 0.01$ when compared to control; and (**) denotes $P < 0.001$ when compared to control, using one-way ANOVA, followed by Dunnett's test.

CDNB did not inhibit the relaxation responses of two non-nitrate vasodilators, viz. isoproterenol and nifedipine. At 160 μM, CDNB in fact increased the relaxation responses toward 1.2×10^{-7} M isoproterenol (control 57.8 ± 7.4%, CDNB 90.0 ± 6.3%, N = 7, $P < 0.001$) and 2×10^{-7} M nifedipine (control 65.4 ± 5.39%, CDNB 85.0 ± 6.9%, N = 5, $P < 0.001$).

NO generation from NTG in intact vascular preparations of the bovine coronary artery was readily measured by RCD. BSP (at 200 μM) did not produce a signifcant effect on the relaxant effects of NTG, nor did it affect NO generation from NTG in the preparation (Fig. 3). In contrast, CDNB (at 160 μM) significantly inhibited the relaxation response toward NTG as well as NO generation (Fig. 3, $P < 0.01$ compared to the respective controls).

Preincubation of bovine coronary rings with 0.44 mM NTG for 1 hr produced a rightward shift of the concentration–response curve (Fig. 4). NTG-tolerant rings were about 162 times less sensitive than control (EC_{50} values were $3.18 \pm 5.98 \times 10^{-7}$ for control and $5.13 \pm 3.02 \times 10^{-5}$ M for tolerant rings, respectively, $P < 0.01$). When control and tolerant rings were challenged with 366 μM NTG in separate experiments for NO measurements, tolerant rings produced 67.9% of NO relative to control (Fig. 5B, $P < 0.01$). This compared to a decrease to 71.8% in relaxation response at an agonist concentration of 316 μM NTG (Fig. 5A, $P < 0.01$). NO recoveries from control and tolerant ring preparations were not different (NO AUC values for control and tolerant rings were 278 ± 57 and 267 ± 45.1 pmol · 100 μL headspace^{-1} · min · mg protein^{-1}, respectively, N = 4, $P > 0.05$), indicating that the decreased NO concentration in headspace of NTG-tolerant preparations was not due to reduced distribution of NO from the tissue to the headspace.

Reduced NO production could be observed in

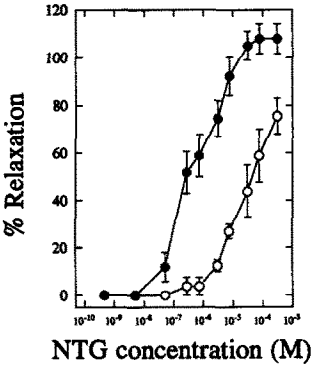

Fig. 4. Development of NTG-induced vascular tolerance. Each point represents the mean ± SD of three separate determinations. Key: (●) control; and (○) tolerant.

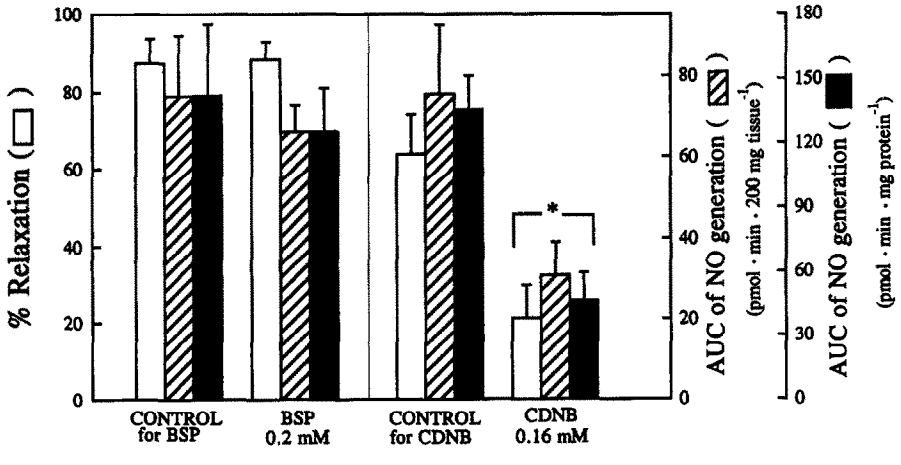

Fig. 3. Effects of BSP and CDNB on NTG-induced relaxation response (□), and on NO generation from vascular microsomes (■) and from intact vascular rings (▨). Data are means ± SD. Conditions were described under Materials and Methods (N = 3–4 in each observation). Key: (*) denotes statistical difference compared to corresponding control ($P < 0.01$).

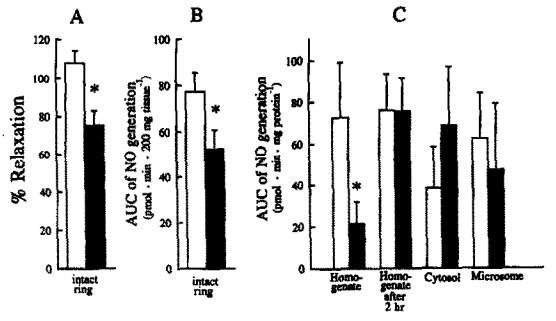

Fig. 5. Effect of tolerance development on relaxation and NO generation from various cellular preparations. For all panels, (*) denotes statistical difference ($P < 0.01$) compared to corresponding control. Key: (□) control; and (■) tolerant. Panel A: Effect of tolerance on NTG-induced relaxation at 316 μM NTG (N = 5). Panel B: Effect of tolerance on NO generation from 366 μM NTG (N = 5). Panel C: Effect of tolerance on NO generation from 366 μM NTG in homogenates (N = 6), cytosol (N = 10) and microsome (N = 10). In all panels, data are expressed as means ± SD.

homogenates of tolerant rings compared to controls, but this difference was no longer present in the two major subfractions further obtained from the homogenate, viz. the cytosolic and microsomal fractions (Fig. 5C). Since preparation of these subcellular fractions required about 2 hr at about 4°, we examined whether this time delay and temperature condition might have obliterated the difference in NO production due to tolerance development. Our results (Fig. 5C) indicated that maintaining the homogenate preparation for 2 hr at 4° indeed removed the difference in NO production between tolerant and non-tolerant arterial rings previously observed in fresh homogenates.

DISCUSSION

Several experimental findings strongly suggest that the vasodilating activity of NTG is mediated by its biotransformed product, NO, in the vasculature. First, both NTG and NO act intracellularly by activating guanylate cyclase, and elevating cyclic GMP levels [1]. A substance such as methylene blue that inhibits NTG action also inhibits NO action [10]. Second, the vasodilating action of NTG is intimately linked to its metabolism to its dinitrate metabolites. Brien *et al.* [13] have shown that the time course of dinitrate formation from NTG in vascular segments closely parallels that of vasodilation. Tolerance development to the vasodilating effects of NTG is accompanied by reduced formation of dinitrate metabolites [2, 14]. Third, we recently demonstrated that NO could be generated enzymatically from NTG in incubations of subfractions of bovine coronary smooth muscle cells [3, 4].

Despite these strong indications, no direct evidence has been presented until recently to demonstrate that perturbations of NTG action are accompanied by parallel changes in NO generation. By monitoring the coronary effluent of a Langendorff preparation of rabbit hearts, Forster *et al.* [15] were able to show that development of nitrate tolerance was accompanied by reduced NO release. However, the corollary proof, viz. that perturbations of NO production would produce parallel changes in NTG-induced relaxation, is not available from the literature.

We have an opportunity to examine the validity of this corollary proof because we have shown recently that, although GSTs are not primarily involved in the metabolic activation of NTG to NO in the microsomes of bovine coronary artery smooth muscle cells, two GST-related substances showed different effects on the microsomal generation of NO from NTG [4]. Thus, a known GST inhibitor, BSP (up to 800 μM), has no apparent effect on NO generation from NTG, while CDNB, a known GST substrate, shows significant inhibition of NO-generating activity from NTG in vascular microsomes [4]. The divergent effects of these two substances on NO production from NTG allowed us to examine whether the relaxant effects of NTG are similarly affected.

While the extent of NO production from NTG from various subcellular fractions can be measured easily [3, 4], the literature has not reported an example wherein NO production from NTG with intact vascular segments has been directly quantitated. We showed that by using a larger quantity of coronary rings (200 mg), direct measurement of NO from micromolar concentrations of NTG can be accomplished (Fig. 3). We showed that, consistent with our observation with vascular microsomes, BSP did not affect NO production from NTG in intact vascular rings at BSP concentrations of 100 and 200 μM. This lack of effect on NO production was paralleled by a similar lack of effect on NTG-induced relaxation at these BSP concentrations. The latter finding disagreed with that reported earlier by Yeates *et al.* [16] who showed that 100 μM BSP inhibited the *in vitro* relaxation of rabbit aorta by NTG. It is not clear why the inhibitory effect of BSP could not be observed in this study. Since we did not directly measure intracellular BSP concentration in our study, it is possible that inhibitory intracellular BSP concentrations were not achieved. Another possible reason could be due to the differences in the tissue preparation, including a difference in the species used (viz. bovine vs rabbit) and the source of tissue (viz. coronary vs aortic), since the vasodilating properties of blood vessels from different origins and species are known to be widely divergent [17]. Our data, however, are consistent with a recent study by Lau and Benet [18], who also could not reproduce the findings of Yeates *et al.* [16].

In contrast, in vascular rings challenged by NTG, CDNB inhibited both NO production and relaxation (Figs. 2 and 3). Inhibition of NTG-induced relaxation was significant at CDNB concentrations above 40 μM (Fig. 2); this inhibition appeared irreversible since removal of CDNB and washing of the rings did not restore sensitivity toward NTG within 15 min. The mechanism of CDNB inhibition of both the metabolic

activation and pharmacologic activity of NTG is not known, but is unlikely due to non-specific damage to the mechanical properties of the vascular rings, since at identical CDNB concentrations, the relaxant responses toward two non-nitrovasodilators, viz. isoproterenol and nifedipine, were not only undiminished, but showed enhanced vasodilation. These effects, however, could be abolished by removing CDNB and washing the rings, in the same manner described for the NTG/CDNB experiment (data not shown). Thus, the apparently irreversible inhibitory properties of CDNB appeared specific for NTG.

Production of *in vitro* nitrate tolerance was accomplished by incubating the bovine vascular rings at 0.44 mM NTG for 1 hr, as evidenced by the rightward shift of the concentration–response curve (Fig. 4). This decreased relaxation reactivity toward NTG was accompanied by a similar decrease in NO production from intact rings (Fig. 5, A and B). The observed difference in NO production between tolerant and non-tolerant vessels remained evident when the tissues were freshly homogenized and challenged with NTG, but not when the homogenates were left to stand for 2 hr at 4°. Separation of the homogenates into a cytosolic fraction and a microsomal fraction, which required approximately 2 hr, also appeared to abolish the difference in NO production between tolerant and non-tolerant vessels previously observed in fresh tissue homogenates. These observations suggested that some degree of reversal of tolerance might occur *in vitro*, consistent with previous literature findings [19, 20]. These results pointed out the difficulty in studying metabolic activation of NTG in subcellular fractions as a function of tolerance development since the time required to prepare these subfractions may obliterate any potential difference that might be present in freshly prepared samples.

It is recognized that the NTG concentration used in the NO-generation experiment (viz. 366 μM) was about 3 orders of magnitude higher than the normal EC_{50} of NTG in inducing vascular relaxation. The need to use this higher concentration in the biochemical experiment was most likely caused by a dilution factor inherent in the headspace NO assay that we used. The small amount of NO needed to elicit relaxation was probably generated within the smooth muscle cell, in close promixity to the effector enzyme guanylate cyclase. We were unable to measure this intracellular NO concentration, and had to rely on measuring the bulk NO concentration liberated into the headspace, thus losing considerable sensitivity in NO quantitation in the experiment. Nevertheless, in experiments where relaxation and NO generation were compared (Figs. 3 and 5), similar NTG concentrations were used in both measurements.

In conclusion, in the three systems that we have examined, the results are confirmatory of the view that NO production from NTG is responsible for its vasodilating activity. Thus, with BSP, no effect on NO production was anticipated and no effect on NTG-induced relaxation was observed. With CDNB, inhibition of NO production was planned (based on our experience with vascular microsomes), and indeed both vascular metabolic activation to NO and vascular reactivity were decreased. With tolerance development, the reduced vascular activity was expected to be accompanied by a decrease in NO production, and this was indeed observed.

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