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Specific Binding of Nitroglycerin to Coronary Artery Microsomes

EVIDENCE OF A VASCULAR NITRATE BINDING SITE

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ABSTRACT. The vasodilating action of organic nitrates, such as nitroglycerin (NTG), is thought to be mediated through metabolic conversion to nitric oxide (NO) in vascular smooth muscle. Although the pertinent enzyme(s) that carries out this crucial step has not been identified, previous studies have shown that the primary enzymatic site is located within cellular membrane fractions. In these studies, we examined the binding of [^{14}C]NTG to microsomal fractions from bovine coronary arteries. Specific binding was linearly related to protein concentration, and binding equilibrium was reversible, reached equilibrium within 1 hr, and remained stable for 4 hr at 25°. Competition experiments with unlabeled NTG demonstrated the presence of two binding sites of differing affinities (high-affinity site: B_{max} 24.1 ± 0.9 pmol/mg protein, K_d 554 ± 22 pM; low-affinity site: B_{max} 79.0 ± 2.9 pmol/mg protein, K_d 151 ± 3 μM). Both of the thiol alkylators 1-chloro-2,4-dinitrobenzene and *N*-ethylmaleimide were found to inhibit [^{14}C]NTG binding, as well as enzymatic generation of NO from NTG, in a concentration-dependent manner. Competition of [^{14}C]NTG was also observed with five other organic nitrate vasodilators, and the degree of competition was linearly related to the *in vitro* vaso-relaxing potencies of these agents. Parallel experiments also showed that in the absence of thiol cofactor, the enzymatic production of NO from NTG was antagonized competitively by less potent organic nitrates. Intact blood vessel experiments using rat aorta also showed that the presence of isosorbide dinitrate (ISDN), at concentrations that did not induce relaxation alone, caused a slight but significant shift in the relaxation potency of NTG (EC_{25} 9 ± 2 versus 28 ± 7 nM, in the presence and absence of 0.3 μM ISDN, respectively; $P < 0.05$). These results demonstrate the presence of specific binding of organic nitrates to microsomal proteins in vascular smooth muscle, and the observed binding is apparently related to enzymatic conversion to NO and the vasodilating properties of these compounds. BIOCHEM PHARMACOL 52;4:619–625, 1996.

KEY WORDS. organic nitrates; nitroglycerin; binding; nitric oxide; vascular

Although NTG† is an important vasodilating drug that has been used in the treatment of angina pectoris for over 100 years, its biochemical mechanisms of action have only begun to be unraveled in the past two decades [1–3]. It is now generally believed that the vasoactivity of NTG and other organic nitrates arises from vascular metabolism to NO [1, 2], a potent and short-lived endogenous vasodilating substance [4]. The NO produced then causes stimulation of cytosolic guanylate cyclase and vascular relaxation via a cyclic GMP pathway [3–5].

In 1973, Needleman *et al.* first suggested the possibility of an organic nitrate receptor in vascular smooth muscle [6], but a "traditional" pharmacologic receptor has not been identified. More recently, it has become apparent that the pharmacologic "target" for organic nitrates is one or more enzymes within vascular cells [7]. Previous studies in our laboratory have shown that the predominant vascular enzyme activity is associated with the cellular plasma membrane [8]. The actual identity of this primary enzyme in vasculature is not known, but recently it was partially purified and characterized [9]. This enzyme utilizes a thiol cofactor to metabolize nitrates and liberate NO [9], but apparently it is not glutathione *S*-transferase [10]. Here we have attempted to study the binding of a radiolabeled substrate (i.e., [^{14}C]NTG) to bovine coronary artery microsomes in the absence of thiol cofactor, in an attempt to probe the characteristics of this enzyme system further. Parallel studies examining nitrate conversion to NO and vasorelaxation were also conducted to evaluate the pharmacologic relevance of the observed binding phenomena.

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† *Abbreviations:* NTG, nitroglycerin; NO, nitric oxide; CDNB, 1-chloro-2,4-dinitrobenzene; NEM, *N*-ethylmaleimide; ISDN, isosorbide dinitrate; PMSF, phenylmethylsulfonyl fluoride; IS5MN, isosorbide 5-mononitrate; IS2MN, isosorbide 2-mononitrate; IIMN, isoidide mononitrate; and IMMN, isomannide mononitrate.

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MATERIALS AND METHODS

Bovine Coronary Artery Microsomes

Microsomes were prepared as previously described by Chung and Fung [8]. Circumflex coronary arteries were

isolated from bovine hearts, cleaned, and everted. Intima and media layers of smooth muscle were dissected, minced, and weighed. Homogenates were made in 5 vol. of 250 mM sucrose/0.5 mM PMSF buffer at 4°. Homogenates were centrifuged at 10,000 *g* for 10 min, and the supernatant was spun at 210,000 *g* for 60 min. Then the microsomal pellet was washed once in buffer by resuspending and centrifugation. This preparation contained membrane-bound proteins from both intracellular and plasma membranes. Pellets were stored at −70° for no more than 2 weeks before use. Preliminary studies showed that enzymatic activity (conversion of NTG to NO) was maintained with these storage conditions. Microsomal pellets were freshly resuspended on each experimental day in sucrose/PMSF buffer to a concentration of 5 mg protein/mL and stored on ice until used. Aliquots were then diluted in Tris buffer just prior to use. Protein concentrations were determined by the method of Lowry *et al.* [11].

[^{14}C]NTG Binding Studies

Competition experiments were conducted using [^{14}C]NTG and six unlabeled organic nitrates (NTG, ISDN, IS5MN, IS2MN, IIMN, and IMMN). These compounds were chosen because they have been shown to have a wide range of vascular and hemodynamic potencies [12], and they were available in our laboratory at high purity. [^{14}C]NTG (sp. act. 52 mCi/mmol) was donated by G. D. Searle (Chicago, IL), and was purified to 99.7% by thin-layer chromatography prior to use [13]. Unlabeled NTG (1.0 mg/mL, 4.44 mM in 5% dextrose) was donated by Schwarz Pharma (Monheim, Germany). All other solutions of organic nitrates were made in 5% dextrose.

Mixtures of [^{14}C]NTG, unlabeled organic nitrates, and microsomal protein (1 mg protein in 1 mL total volume) were incubated in 25 mM Tris buffer, pH 8.0, at 25 ± 1°. This pH has been shown to be optimal for enzymatic activity [9], and this temperature was chosen because it afforded better control during the incubation and transfer steps. At specified times, 0.5 mL of the mixture was vacuum-filtered (Millipore 1225 sampling manifold and HATF nitrocellulose filters, 0.45 μm pore size, Bedford, MA), and washed rapidly with 3 × 3.5 mL of ice-cold Tris buffer. Filters were solubilized in 10 mL of scintillation fluid, and radioactivity was counted with quench correction (Packard LSC, model 1900CA). Preliminary experiments were conducted to characterize the time–course and linearity of NTG binding to coronary microsomes using [^{14}C]NTG (524 nM). In other experiments, NTG binding affinities and capacities were studied by displacement of [^{14}C]NTG (655 nM) using unlabeled NTG (twenty-three different concentrations ranging from 1.0 pM to 2.2 mM). Curve-fitting of the competitive binding data was performed using nonlinear regression analysis (Inplot, Graphpad, San Diego, CA). Separate studies were also conducted to evaluate the relative abilities of other organic nitrates to displace [^{14}C]NTG at a single concentration. Microsomal protein (1 mg in 1 mL, pH 8.0, 25°) was incubated with [^{14}C]NTG (655 nM) in the absence or presence of unlabeled organic nitrates (600 μM). After 1 hr, samples were rapidly filtered and washed, and the amount of bound radiolabel was quantified. Data are presented as percent inhibition of [^{14}C]NTG binding at this single concentration of competitor. In all experiments, nonspecific binding was determined in the presence of 2.2 mM unlabeled NTG, and represented 17 ± 2% of the total radioactivity detected (mean ± SD, N = 16).

Enzymatic NO Production

Production of NO from organic nitrates was detected with a Redox-Chemiluminescence Detector (RCD, Seivers Research, Boulder, CO) using methods similar to those previously described [14]. Microsomal protein (1 mg in 2 mL Tris buffer, pH 8.0) was incubated with NTG and/or other unlabeled organic nitrates in sealed reaction vials and stirred continuously at 25°. Superoxide dismutase was added (100 U/mL) to stabilize NO in solution and enhance partitioning into vial headspace [8]. The amount of NO in the vial headspace was determined by vacuum-sampling the entire headspace after 2 or 4 hr of incubation. The longer times of incubation used for these enzyme experiments relative to the rapid vascular actions of NTG (2 or 4 hr of incubation versus an onset of relaxation in minutes) were required for reliable measurement of NO production and to allow for equilibration of NO gas into the vial headspace. Preliminary experiments showed that cumulative enzymatic NO production from 150 μM NTG was linear for at least 5 hr, suggesting the absence of biochemical tolerance development under the conditions used. The RCD instrument was calibrated daily using known standards of NO gas, and the lower limit of detection was 0.4 pmol of NO injected. Recovery of NO from protein solutions was assessed by injecting volumes of NO-saturated water into sealed vials containing protein, and was determined to be 64 ± 2%. Absolute NO recovery from aqueous solutions without protein was 99 ± 3% (N = 5), and enzymatic NO production was calculated from the difference observed between samples with protein versus without protein, after correction for recovery.

Vascular Relaxation Experiments

Isolated blood vessel relaxation experiments were conducted in a manner similar to the methods previously described [15]. Rat abdominal aorta was excised, mounted on force transducers, and incubated in Krebs-bicarbonate buffer, pH 7.4, at 37°. Vessel segment tension was recorded on a physiograph (Grass Instruments, Quincy, MA). Tissues were preconstricted with 0.5 μM phenylephrine, and cumulative NTG concentration–response curves were determined. Nonlinear curve-fitting was performed with the data from each vessel segment, using a sigmoidal E_{max} model (PCNONLIN; SCI Software, Lexington, KY).

RESULTS

Preliminary experiments demonstrating the time-dependence and linearity of [^{14}C]NTG binding to bovine coronary artery microsomes are shown in Fig. 1. When 1 mg of microsomal protein was incubated with 524 nM [^{14}C]NTG, time-dependent binding was observed and equilibrium was established within 1 hr (Fig. 1) and maintained throughout 4 hr. This binding was also shown to be linearly related to protein concentration (inset of Fig. 1).

Figure 2 shows the competitive displacement of [^{14}C]NTG by unlabeled NTG. Two binding sites were apparent. To provide estimates of the high- and low-affinity binding constants, all of the available data were collectively fit to a two-site model. A total of 90 data points were used for this fitting process, yielding the following results: high-affinity site, K_d 554 ± 22 pM, B_{max} 24.1 ± 0.9 pmol/mg protein; low-affinity site, K_d 151 ± 3 μM, B_{max} 79.0 ± 2.9 pmol/mg protein.

Figure 3 shows the effects of two known inhibitors of organic nitrate bioconverstion of [^{14}C]NTG binding and NO production from unlabeled NTG. The presence of CDNB or NEM caused concentration-dependent inhibition of NO production from 150 μM unlabeled NTG, and NEM was more potent than CDNB in this respect. In parallel experiments, the total binding capacity of microsomal protein was examined in the absence and presence of the same inhibitors, and apparent maximal binding was assessed by the presence and absence of 2200 μM unlabeled NTG. Similar to the parallel NO production experiments, the presence of each inhibitor caused concentration-dependent reductions in the apparent [^{14}C]NTG B_{max}.

Figure 4A shows the displacement of [^{14}C]NTG binding by various other organic nitrates. NTG (600 μM) inhibited the radioligand binding by 92 ± 2%, whereas the other

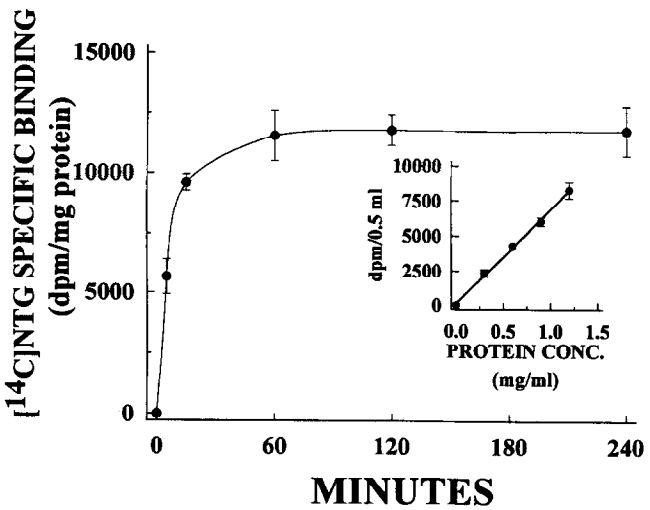

FIG. 1. Time-dependence and linearity (inset) of [^{14}C]NTG binding to bovine coronary artery microsomes (means ± SD, N = 5). Specific binding was linearly related to protein concentration (inset, r = 0.988, P < 0.01).

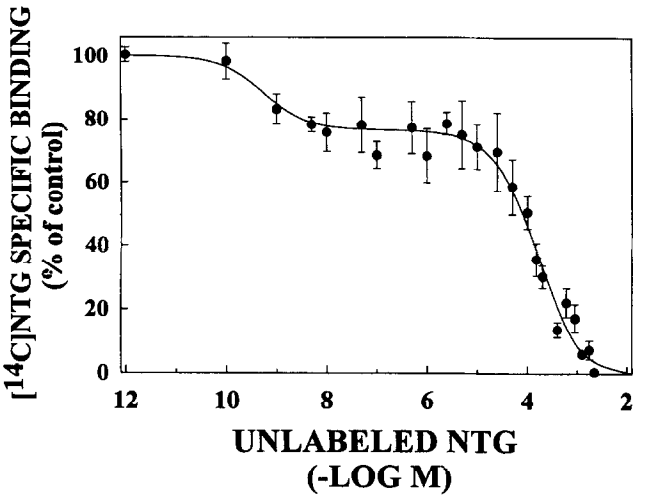

FIG. 2. Competitive binding of [^{14}C]NTG (655 nM) and unlabeled NTG (means ± SD, N = 5–9). Results are expressed as a percentage of specific binding (determined by the presence of 2200 μM NTG). Specific [^{14}C]NTG binding was 11,530 ± 1,400 dpm/mg protein. A total of 90 data points were used for the fitting process.

organic nitrates could also displace [^{14}C]NTG, but to a lesser extent. In Fig. 4B we examined the relationship between the relative affinities of organic nitrate binding at this single concentration of 600 μM to the known relative vascular potencies of these agents; the latter of these parameters, as presented by the rat vascular EC_{50} values, were taken from previously published work from our laboratory [12, 15]. A statistically significant negative correlation was observed between inhibition of [^{14}C]NTG binding and nitrate vascular EC_{50} (Spearman nonparametric correlation analysis, P < 0.01), even though the binding data were obtained with bovine coronary microsomes and the potency data were obtained with rat abdominal aortae.

Metabolic experiments were also conducted to examine possible competitive enzyme inhibition of organic nitrates (Fig. 5). Microsomal protein (1 mg in 2 mL with SOD 100 U/mL) was incubated with NTG (150 μM), ISDN (1500

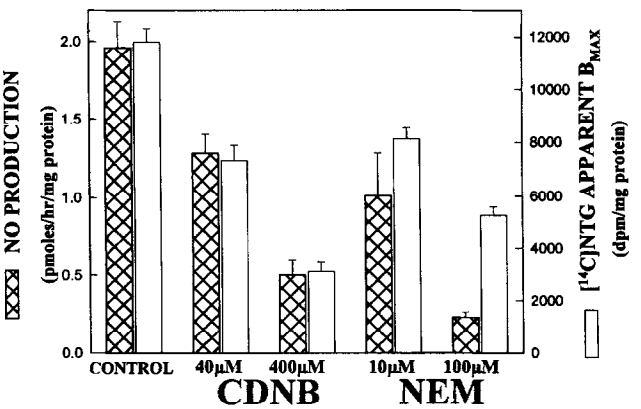

FIG. 3. Inhibition of microsomal NO production from NTG and [^{14}C]NTG binding (means ± SD, N = 5).

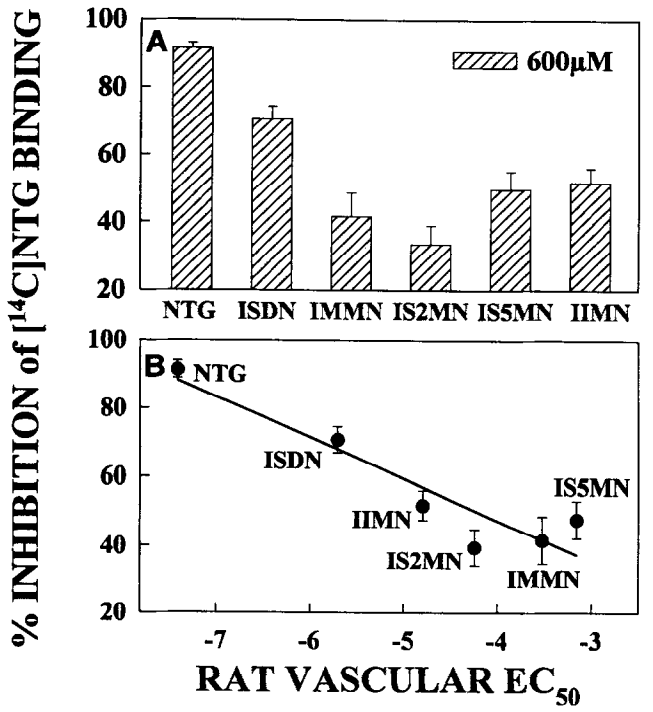

FIG. 4. (A) Displacement of [^{14}C]NTG binding by other unlabeled organic nitrates (means ± SD, N = 6). Specific [^{14}C]NTG binding was 11,530 ± 1,400 dpm/mg protein. (B) Relative abilities of the unlabeled nitrates to inhibit [^{14}C]NTG binding (i.e. apparent relative affinities) correlated to their relative potencies in rat vascular tissues ($P < 0.05$, Spearman non-parametric correlation analysis).

μM), or IS5MN (1500 μM), and the amount of NO in the headspace was measured at 4 hr. ISDN and IS5MN produced less NO than NTG, despite incubation at 10-fold higher substrate concentrations. Co-incubation of NTG (150 μM) with these less active organic nitrates (at a 10-fold excess) led to less metabolic production of NO than when NTG was incubated alone ($P < 0.05$). These results are consistent with competitive binding to the NO generating enzyme for organic nitrates (i.e. competitive substrate inhibition).

Figure 6 shows the *in vitro* vascular responses to NTG in the presence and absence of ISDN. Segments of rat abdominal aorta were precontracted with phenylephrine, and then cumulative NTG concentration–response curves were produced in the absence or presence of ISDN. ISDN (or 5% dextrose vehicle) was added to the bath at a final concentration of 0.3 μM 5 min prior to adding NTG to the bath. This ISDN concentration is only 5% of its known EC_{50}, and caused no measurable vasorelaxation. The initial contraction prior to NTG was not different between the two treatment groups (control 1.21 ± 0.28 g tension; ISDN-pretreated group 1.20 ± 0.23 g, NS). The presence of ISDN at a sub-effective concentration caused slight but statistically significant reductions in the relaxation potency of NTG (NTG alone, EC_{25} 9 ± 2 nM, EC_{50} 21 ± 4 nM; NTG in the presence of ISDN, EC_{25} 28 ± 7 nM, EC_{50} 53 ± 7 nM, $P < 0.05$ for both parameters), but the maximal relaxation was unaffected (E_{max} 103 ± 4 versus 95 ± 3%, respectively, NS).

DISCUSSION

Over two decades ago, Needleman *et al.* postulated a specific receptor site for organic nitrates in vascular smooth muscle cells [6], but since that time no such receptor has been identified or characterized. More recently, the vasorelaxant action of organic nitrates has been shown to co-

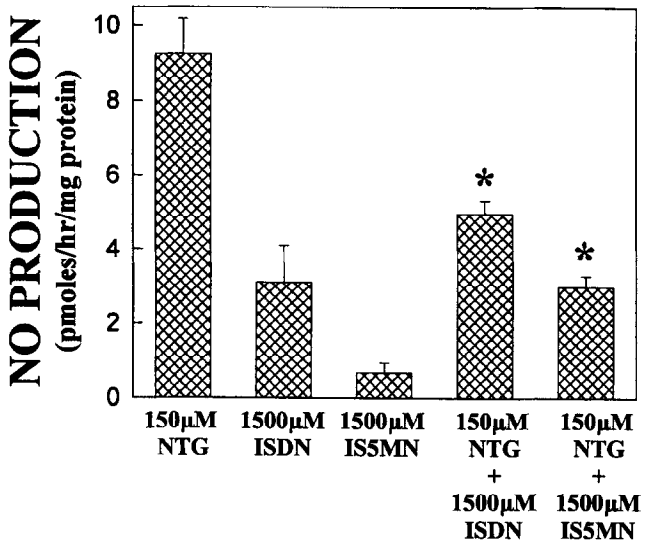

FIG. 5. Competitive inhibition of NO production from NTG by less potent organic nitrates (means ± SD, N = 5). Key: (*) statistically significant from NTG alone ($P < 0.05$).

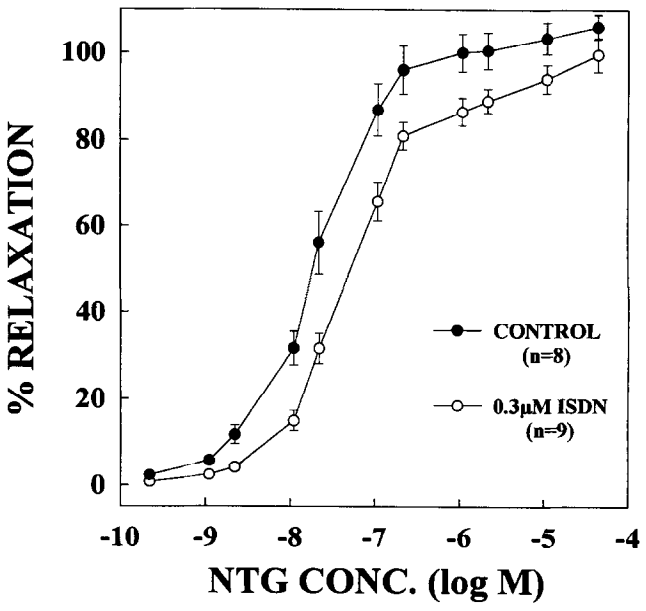

FIG. 6. Antagonism of NTG relaxant effects in the presence of 0.3 μM ISDN. Values are means ± SD.

incide with metabolism in vascular tissues [5], suggesting that the pharmacologic target for these agents is a cellular enzyme rather than a traditional receptor site. It is now generally believed that organic nitrate action is governed by vascular conversion to NO, and subsequent relaxation via a cyclic GMP-dependent process. The enzyme that metabolically converts organic nitrates to NO has been examined by us and others, but its identity remains controversial [16]. This has been complicated by the fact that there are probably at least two metabolic pathways involved: one in which NO is produced (thus, a "pharmacologically productive" pathway) and a second metabolic pathway producing nitrite and/or nitrate ions without substantial liberation of NO [17]. A further complication is the fact that the presence and relative abundance of each enzyme system may be organ-specific, and results from liver homogenate studies may not be reflective of vascular processes [17].

Chung and Fung have shown that in bovine coronary artery smooth muscle the primary NO-generating activity from nitroglycerin is membrane bound [8], and that all organic nitrates apparently utilize a common enzyme in vascular microsomes [18]. Enzymatic activity can be enhanced by the addition of reduced thiol compounds, is not affected by detergent solubilization, and has a pH optimum at 8.0 [9]. Here we have asked if specific binding of radiolabeled nitrate to vascular microsomes could be detected, using a substrate with high specific activity (52 mCi/mmol) and in the absence of thiol cofactor. Exogenous thiol was excluded from our experimental conditions to avoid chemical (non-enzymatic) conversion of nitroglycerin, and to allow for the study of nitrate–protein interactions. We also conducted the binding and enzyme experiments at 25° (room temperature). In preliminary studies we found the binding process to be reduced and more variable at 37°, possibly due to the more rapid rates of nitrate–protein interactions (i.e. a less stable substrate–enzyme complex). To account for this temperature condition, we conducted parallel NO production studies also at 25°. Previous studies by Booth *et al.* [19] have shown that the biotransformation of NTG to NO is reduced at lower temperature (27° in their studies), and this is associated with a parallel reduction in vascular potency. These findings, taken together with our observations, support the hypothesis that the observed binding of NTG (and competition by other organic nitrates) is related to their biochemical mechanism of vasorelaxation.

Competitive binding of unlabeled NTG provided evidence of two binding sites with differing affinities. In contrast, previous enzyme studies with the same tissue preparation have shown the presence of only one V_{max} and K_m for the metabolic conversion of NTG to NO [8]. It is possible that the apparent high-affinity binding site we have observed is not related to enzymatic conversion. Interestingly, Torfgard *et al.* [20] have described a biphasic concentration–effect curve for NTG and postulated that NTG has a partly unique mechanism of vascular smooth muscle relaxation that distinguishes this compound from other related organic nitrates. The high-affinity relaxation component is apparently inhibited by pertussis toxin, suggesting that this interaction may be with a regulatory protein [21]. Further studies examining this apparent high-affinity binding component may help explain this activity of NTG.

Similar to other previous studies [22], both CDNB and NEM inhibited the production of NO from NTG. In parallel experiments, we also found that these inhibitors of metabolic conversion also decreased the maximal binding of [^{14}C]NTG in a concentration-dependent manner. Since these agents are known to alkylate free sulfhydryl (–SH) groups [23], our results indicate the presence of one or more –SH groups at or near the active site, which is required for both substrate binding and enzyme activity. Others have also shown the requirement of available –SH groups for the vasoactivity of organic nitrates [24], and oxidative loss of these sites has been suggested as a mechanism of nitrate vascular tolerance [25, 26].

At a single concentration (600 μM), five other organic nitrate vasodilators showed abilities to displace [^{14}C]NTG binding. At this concentration unlabeled NTG produced 90% inhibition, and it was anticipated that the less potent nitrates would thus provide measurable displacement. Further studies to determine binding affinities of each nitrate were not conducted because of limited availability of protein and radiolabel. The relative affinities of the organic nitrates (as determined by percent inhibition of [^{14}C]NTG binding at 600 μM) were found to correlate with their relative vascular potencies. While the vascular EC_{50} values for this relationship were taken from rat aorta data, rather than bovine tissues, the relative *in vitro* potencies of nitrates have been shown to be generally consistent across species [27]. These results are consistent with the concept that all organic nitrates utilize the same enzyme system for metabolic NO production [18], and that their relative *in vitro* potencies are related to their respective affinities for this interaction.

Since we observed competitive displacement of [^{14}C]NTG binding by other less potent organic nitrates, we tested whether competitive antagonism of NO production would also be observed. In the absence of additional thiol, 150 μM NTG produced readily detectable amounts of NO, but 10-fold higher concentrations (1500 μM) of ISDN and IS5MN were required to produce detectable amounts. Additionally, metabolic NO production from NTG was inhibited significantly ($P < 0.05$) by co-incubation with 10-fold excess ISDN or IS5MN. These results are consistent with our results on competitive binding and further support the hypothesis of competitive substrate inhibition. Does this apparent competitive antagonism in isolated microsomes produce a change in pharmacologic activity? We attempted to test this hypothesis using isolated rat tissues, and found slight but statistically significant antagonism of NTG vasorelaxation in the presence of a sub-effective concentra-

tion of ISDN. In contrast to our findings, Bennet *et al.* [28] found no evidence of organic nitrate competitive antagonism. Using an isobolographic method, with fixed ratio mixtures of ISDN and IS5MN, these authors concluded that "IS5MN, the major metabolite of ISDN, is not an antagonist of ISDN at a 'nitrate receptor' " [28]. Our studies show that the binding characteristics and NO conversion for ISDN and IS5MN are quite similar. Because of these similar biochemical characteristics, and their similar vascular potencies, it is possible that ISDN and IS5MN may not appear antagonistic. Our ability to detect slight antagonism may have been related to the greater potency differences among the two nitrates we employed (NTG vascular EC_{50} 28 nM versus ISDN 60 μM in this study). Our *in vitro* vascular results are generally consistent with the observed behavior of NTG binding and metabolism using bovine vascular microsomes (namely competitive antagonism of NTG effects by less potent organic nitrates). Since NTG metabolism leads to the production of less potent but pharmacologically active metabolites, it is possible to speculate that accumulation of these metabolites may reduce the potency of NTG via competitive antagonism and contribute to NTG tolerance development. Further experiments are needed to confirm or disprove this speculation.

Previous studies have not been supportive of the concept of a nitrate-receptor. For example, Kawamoto *et al.*[29] attempted to measure radioligand binding using tritiated NTG and whole homogenate and subfractions of bovine pulmonary vein, and detected no binding. Many differences exist between the methods we used here and those of Kawamoto *et al.*, including the radioligand employed (^{14}C- versus ^{3}H-labeled NTG), the tissues studied (bovine coronary artery versus pulmonary vein), and the temperatures studied (25° versus 4° and 37°). In preliminary experiments, we also found that nitrocellulose filters, but not glass fiber filters (used by Kawamoto *et al.* [29]), provided excellent retention of our vascular microsomal proteins and gave more reliable binding results.

The actual identity of the enzyme(s) responsible for nitrate conversion to NO in vascular tissues is not established, but a cytochrome P450 enzyme, a glutathione S-transferase (GST), and a plasma membrane-bound enzyme yet to be named have all been suggested as possible candidates [16, 17]. The studies we present here do not confirm or rule out any of these enzyme possibilities, but provide direct evidence of an interaction between nitrates and microsomal protein(s). Although an inhibition of binding and enzyme activity was observed with NEM and CDNB (two classical GST inhibitors), previous studies from our laboratory have shown that the pertinent microsomal enzyme is not likely to be GST, and these inhibitors are not entirely specific [10]. Using these thiol alkylating agents, and various other organic nitrates, we found that perturbations in [^{14}C]NTG binding were paralleled by changes in NO production. Thus, the binding appears to be a component (and perhaps a prerequisite) of organic nitrate bioactivation. Whether the observed binding is the exclusive component of nitrate activity is unknown at this time, but these techniques may provide a useful approach for the identification of the key enzyme(s) and a further understanding of the biochemical steps involved.

In conclusion, we have examined the binding characteristics of [^{14}C]NTG and other organic nitrates to bovine coronary vascular microsomes. The observed binding was reproducible and was inhibited by known thiol alkylators. In addition, competitive binding was observed with other organic nitrates, and binding was apparently related to metabolic NO production. These phenomena, therefore, appear to be related to the biochemical mechanism and vasorelaxant effects of these agents. Further studies using such methods may help to better define and characterize the protein(s) responsible for nitrate-induced vascular effects.

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