THIOL AGENTS SEPARATE NITRIC OXIDE FORMATION FROM VASODILATION INDUCED BY GLYCERYL TRINITRATE

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ABSTRACT:

The role of nitric oxide (NO) and thiol-containing compounds in glyceryl trinitrate (GTN)-induced vasodilation was investigated using the thiol-alkylating agent N-ethylmaleimide (NEM). Bovine pulmonary artery (BPA) rings were submaximally contracted with K+ and exposed to increasing concentrations of GTN after a 30-min incubation with 50 μM NEM. NEM decreased maximal relaxation (10 μM GTN) by 20%, compared with controls. Treatment with 5 mM L-cysteine for 30 min before incubation with 50 μM NEM (protection protocol) prevented this decrease in GTN-induced relaxation, but 5 mM D-cysteine did not. Treatment of BPA rings with 5 mM L-cysteine after NEM treatment (reversal protocol) did not reverse the effect of NEM to decrease relaxation inducible by GTN. NO production from 30 μM GTN (chemiluminescence-headspace gas method) in the presence of BPA strips was 46.7 ± 19.4 pmol NO/g tissue after 10 min of incubation and 76.4 ± 10.4 pmol NO/g tissue after 20 min. After a 30-min incubation with 50 μM NEM, NO was not detected at either time point. NO production from GTN by BPA strips, with either the protection or reversal protocol, was elevated approximately 2-fold at both time points, compared with controls. No increase in NO production from GTN was observed at either time point for tissues treated with 5 mM D-cysteine using the same protocols. These results are consistent with the concept that thiol compounds play a role in the mechanism of GTN-induced vasodilation, but they indicate that the mechanism of action of GTN and other organic nitrates is more complex than their acting as immediate prodrugs of NO.

The mechanism of action of organic nitrate-induced relaxation of VSM has been subjected to much investigation in the past two decades, but it is still not fully understood. According to a widely expressed hypothesis, organic nitrates are biotransformed to NO, which activates soluble guanylyl cyclase, thereby causing an increase in cGMP formation and subsequent vasodilation (Ignarro et al., 1981). On the other hand, a number of observations inconsistent with the direct formation of NO, as stated, have been reported. For example, our laboratory was unable to measure the formation of NO from GTN in vascular tissue until relaxation was almost fully developed (5 min), whereas NO formation from sodium nitroprusside, 3-morpholinosydnonimine, or S-nitrosothioacetilamine was measured at time points coincident with vascular relaxation (0.5 min) (Marks et al., 1995). In another experiment, a sandwich bioassay was established in which rabbit aorta was used to generate an active metabolite from GTN and rabbit taenia coli was used as the detector (Hussain et al., 1996). When a superoxide-generating system was present, relaxations induced by NO itself were blocked but the relaxations induced by GTN were unaffected. This was interpreted to mean that the smooth muscle-relaxing factor formed from GTN by the generator was not NO but perhaps was an NO-containing compound. An explanation for these observations might be found in the reports of Yeates et al. (1985) and Lipton et al. (1993), for example, who suggested that GTN interacts with a thiol to form a vasoactive S-nitrosothiol.

The ability of various thiol-containing compounds to facilitate the conversion of GTN to NO (Feilisch and Noack, 1987) and the requirement for L-cysteine in GTN-induced stimulation of soluble guanylyl cyclase (Ignarro et al., 1981) are consistent with the notion that thiol compounds play an important role in these biotransformation and activation steps and may combine with NO to produce the aforementioned NO-containing compound. Studies demonstrating that in vitro exposure to thiol-containing compounds, such as L-cysteine and NAC, can potentiate the effects of GTN in small canine coronary arteries (Wheatley et al., 1994) and in small porcine coronary arteries (Selke et al., 1991) provide further support for the involvement of thiol compounds in the mechanism of action of GTN. Other studies have shown that NAC also potentiates the effects of GTN when the drugs are administered concurrently to human subjects (Horowitz et al., 1983; Winniford et al., 1986).

In the present study, NEM, a thiol-alkylating agent, was used to study the putative role of NO in mediating the actions of GTN in the relaxation of BPA. The effects of NAC, L-cysteine, and D-cysteine, added before or after incubation of BPA with NEM, were tested to determine the correlation between their effects on GTN-induced vasodilation and NO formation. The present observations indicate that the mechanism of action of organic nitrates is more complex than their acting as immediate prodrugs of NO.

Materials and Methods

Chemicals and Solutions. Krebs’ solution (pH 7.4) contained 120 mM NaCl, 5.6 mM KCl, 1.2 mM MgSO4, 1.2 mM NaH2PO4, 2.5 mM CaCl2, 25
Exposed to Thiol-Modulating Agents. BPA rings were individually suspended in tissue baths using wire hooks and polyester sutures connected to Grass force-depolarizing solutions. The BPA rings were moved by gently rubbing the luminal surface of the rings or strips with a cotton-tipped applicator moistened in Krebs’ solution. Removal of the endothelium was confirmed by the absence of relaxation in response to acetylcholine (10 nM to 10 μM) in the tissue bath. After being rinsed five times with 10-ml volumes of Krebs’ solution, several rings or strips were used in experiments that day; the remainder of the tissue was refrigerated overnight (12–16 hr) in Krebs’ solution at 4°C and used in experiments the next day. The relaxation of the BPA rings to GTN on the next day (EC50 = 27 ± 19 nM, N = 3) was not different from the relaxation of the BPA rings on the day of tissue collection (EC50 = 23 ± 4 nM, N = 5).

Cumulative GTN Concentration-Response Relationships in BPA Rings Exposed to Thiol-Modulating Agents. Relaxation responses of BPA rings to GTN were determined before and after exposure of the tissue to various thiol-modulating agents. BPA rings were individually suspended in tissue baths using wire hooks and polyester sutures connected to Grass force-depolarizing transducers (Grass model FT03D), which were in turn connected to a Grass model 7 polygraph (Grass, Quincy, MA). The BPA rings were maintained at a tension of 2 g in Krebs’ solution at 37°C and continually aerated with 5% CO2/95% O2. After a 1-hr equilibration period, during which the Krebs’ solution was changed every 15 min, the rings were maximally contracted with 60 mM K+-depolarizing solution and then washed. After a second 1-hr equilibration period, with washes every 15 min, the rings were contracted to 60–80% of maximum using 15–20 mM K+-depolarizing solutions. Then, a cumulative GTN concentration-response profile (0.1 nM to 10 μM) was determined for each BPA ring. The tissues were then washed every 15 min for 1 hr, followed by a 30-min incubation with various thiol-modulating agents. At the conclusion of these incubation periods, the rings were washed twice and submaximally contracted with 15–20 mM K+-depolarizing solutions. A second cumulative GTN concentration-response profile (0.1 nM to 10 μM) was then determined for each BPA ring.

Measurement of GTN-Derived NO Formation in the Presence of BPA Strips Exposed to Thiol-Modulating Agents. NO formation from GTN incubated in the presence of BPA strips was measured using a chemiluminescence-headspace gas method described by Brien et al. (1991). One BPA strip was placed in a 6.2-ml micro-Fernbach flask (Ace Glass Inc., Vineland, NJ) containing 3 ml of Krebs’ buffer, 0.1 ml of 3000 units/ml superoxide dismutase (final concentration, 100 units/ml), 0.1 ml of 0.9 M KCl (final concentration, 30 mM K+), and a micro-stir bar to mix the contents during subsequent steps. The flask was sealed using a rubber-seal sleeve flask (Aldrich Chemical Co., Milwaukee, WI), and the sample was incubated in a water bath at 37°C for 5 min. The contents of the flask were then gassed with a stream of 5% CO2/20% O2/75% N2 for 5 min, after which 0.1 ml of a 1 mM GTN solution was added via injection through the septum (final concentration, 30 μM). Aliquots (400 μl) of headspace gas were obtained, using an airtight syringe, at 10 and 20 min after addition of GTN. These samples were injected into a model 270B NO-chemiluminescence analyzer (Sievers Research Inc., Boulder, CO). The amount of NO in each sample was calculated from the chemiluminescence signal using a NO standard curve, after correction with the appropriate blank. A NO standard curve was prepared before each experiment using aqueous NO standards, as previously described (Brien et al., 1991). Each BPA strip was blotted and weighed at the conclusion of the experiment, and NO formation from GTN was expressed as picomoles of NO formed per gram of tissue.

Results

Effects of NEM on GTN-Induced Relaxation of BPA. Relaxation responses of BPA rings to GTN were examined both before and after treatment of the tissue with the thiol-alkylating agent NEM. Several concentrations of NEM were investigated. Incubation with 50 μM NEM for 30 min attenuated the relaxation induced by 0.1 nM to 10 μM GTN, with a 20% decrease in response to the maximal concentration of GTN studied (10 μM) (fig. 1A). The EC50 for GTN-induced relaxation was not altered by incubation with NEM, nor did incubation alter the response of the tissue to K+. Increasing the concentration of NEM above 75 μM increased the antagonism of GTN-induced relaxation of BPA rings to GTN.
relaxation. However, these concentrations of NEM decreased the responsiveness of the tissue to K⁺ and, therefore, were not used in subsequent experiments. Incubation with 10 μM NEM had no effect on the GTN-induced VSM relaxation. The decreased response of the BPA rings to GTN after exposure to 50 μM NEM did not appear to be the result of the development of tolerance. The relaxation of BPA rings that had been previously exposed to GTN but not to 50 μM NEM (time controls) was unchanged during a second exposure to cumulative additions of GTN (fig. 1B). Incubation of BPA rings with 50 μM NEM for 30 min did not decrease the relaxation induced by 0.1 mM to 10 μM isopropyl-norepinephrine, but it did attenuate the relaxation induced by authentic NO. Thus, in two experiments the mean relaxant effect of 2, 9, and 22 nM NO gas was decreased by 15, 21, and 20%, respectively.

**Effects of Thiol-Containing Compounds on GTN-Induced Relaxation of BPA.** Treatment with Thiol-Containing Compounds before Exposure to NEM (Protection Protocol). After the determination of the first cumulative GTN concentration-response relationship and the 1-hr washout period, BPA rings were exposed to 5 mM L-cysteine or NAC. After 30 min, the tissues were washed twice, incubated with 50 μM NEM for an additional 30 min, and again washed twice before the determination of the second GTN concentration-response relationship. Because the amino acid transport systems preferentially deliver L-isomers of amino acids, D-cysteine was used to examine the stereospecific nature of treatment with thiol-containing compounds. Incubation with 5 mM L-cysteine for 30 min, followed by incubation with 50 μM NEM, did not alter the GTN concentration-response curve. Thus, treatment with 5 mM L-cysteine before exposure to NEM prevented the NEM-induced attenuation of the response of the BPA rings to GTN (fig. 2A). Similar results were obtained with NAC (data not shown). In contrast, incubation with 5 mM D-cysteine for 30 min, followed by 30-min exposure to 50 μM NEM, resulted in a 20% decrease in the response of BPA rings to the maximal concentration of GTN (10 μM) but did not alter the EC₅₀ for GTN-induced relaxation (fig. 2B). The results obtained using D-cysteine were similar to those obtained for treatment with NEM alone (fig. 1A).

**Treatment with Thiol-Containing Compounds after Exposure to NEM (Reversal Protocol).** After the determination of the first GTN concentration-response relationship and the 1-hr washout period, BPA rings were exposed to 50 μM NEM for 30 min, washed twice, and then incubated with one of 5 mM L-cysteine, D-cysteine, or NAC. After 30 min, the tissues were again washed twice and a second GTN concentration-response curve was obtained. Incubation with 50 μM NEM, followed by incubation with 5 mM L-cysteine, resulted in a 20% decrease in the response of BPA rings to GTN at the highest concentration used (10 μM) but did not alter the EC₅₀ for GTN-induced relaxation. Thus, treatment with L-cysteine after incubation with NEM did not reverse the attenuation of GTN-induced vasodilation observed after treatment with NEM alone. Similar results were obtained when NAC or D-cysteine was used in place of L-cysteine.

**Effects of NEM on NO Formation from GTN Incubated in the Presence of BPA.** BPA strips were incubated in the presence of 50 μM NEM, in 3 ml of Krebs’ solution, for 30 min. The Krebs’ solution was maintained at 37°C and continuously aerated with 5% CO₂/95% O₂. After the 30-min exposure to NEM, each tissue was washed twice for use in the NO assay. Control incubations were conducted identically except that NEM was omitted from the Krebs’ solution during the 30-min incubation. This incubation of BPA strips with NEM decreased NO formation from 30 μM GTN, measured after 10 and 20 min, compared with controls that were not treated with NEM (fig. 3). NO was not detected in the headspace gas taken from flasks containing NEM-treated BPA strips in the presence of 30 μM GTN, at either time point. Control samples contained an average of 46.7 ± 19.4 and 76.4 ± 10.4 pmol NO/g tissue after 10 and 20 min of incubation, respectively. Recovery of NO from sealed micro-Fernbach flasks containing known amounts of authentic NO was not altered by the addition of NEM alone or NEM in the presence of BPA strips (data not shown). Thus, alkylation of intracellular thiol groups by NEM significantly decreased NO production from GTN in BPA strips.

**Effects of Thiol-Containing Compounds on NO Formation from GTN Incubated in the Presence of BPA.** Treatment with Thiol-Containing Compounds before Exposure to NEM. BPA strips were incubated for 30 min in buffer containing 5 mM L-cysteine, D-cysteine, or NAC. The tissues were then washed twice and incubated in the presence of 50 μM NEM for 30 min. These solutions were maintained at 37°C and continuously aerated with 5% CO₂/95% O₂ gas during each incubation period, which was similar to the experiments involving NEM alone. After 30 min, the BPA strips were washed twice and prepared for use in the NO assay, as described above. At the 10-min time point, there was a 2.1-fold increase in NO formation from GTN for BPA strips that had been incubated with NAC before exposure to NEM (fig. 3A) and a 1.9-fold increase in NO formation for tissues treated in a similar manner with L-cysteine, compared with BPA strips treated with GTN alone. After 20 min, there was a 2.2-fold increase in NO formation from 30 μM GTN for BPA strips treated with either NAC or L-cysteine and then 50 μM
In a reversal of the previous protocol, BPA strips were first treated with 30 μM NEM (fig. 3A). Thus, increased NO formation from GTN was observed for tissues that had been treated with 50 μM NEM and then exposed to either 5 mM L-cysteine or 5 mM NAC. The increased GTN-derived NO formation was similar in magnitude to that observed for BPA strips that had been treated with 5 mM L-cysteine or 5 mM NAC before exposure to NEM.

Discussion

Earlier studies by our laboratory and others indicated that tissue thiols were necessary for GTN and other organic nitrates to be metabolically activated for the induction of blood vessel relaxation (Hussain et al., 1996; Feelsich and Noack, 1987). The goal of the present work was to explore the relationship between the role of thiols and the putative ultimate drug derived from GTN, i.e. NO. The present observations raise questions about the concept of organic nitrates acting via direct formation of NO, with subsequent activation of guanylyl cyclase.

If the key role of thiols in the metabolic activation of GTN involves participation in the direct conversion of GTN to NO, which then initiates a chain of events leading to vascular relaxation, it would be expected that a good correlation would be observed between the effects of a sulfhydryl reagent such as NEM on relaxation and those on NO formation. This was not borne out, because NO formation from GTN was not detected in the headspace gas taken from BPA strips exposed to the same concentration of NEM that produced only 20% inhibition of GTN-induced VSM relaxation (fig. 1). Assuming that direct NO formation from GTN is the critical event in the metabolic activation of GTN, one might have expected a partial reduction in the quantity of NO measured, rather than complete inhibition, to match the incomplete blockade of GTN-induced relaxation. It might be possible to explain this discrepancy between the prodrug hypothesis and the present observations by invoking differences in the sites of measurement. Thus, the site of the NO effect is inside the smooth muscle cells, whereas the site of NO measurement was outside the cells, in the headspace gas above the physiological solution. The observation that authentic NO-induced relaxation was antagonized by 50 μM NEM treatment is consistent with this interpretation. Nevertheless, the subsequent observations made after the “protection” and “reversal” treatments with thiol agents are more difficult to reconcile and suggest that the prodrug hypothesis may need to be reconsidered.

In the case of the protection experiments, treatment with the L-isomers of cysteine and NAC before NEM treatment resulted in approximate doubling of the amount of NO formation from GTN, whereas there was no increase in sensitivity to GTN reflected in the dose-response curve for GTN. On the basis of the dose-response relationship, it was anticipated that L-cysteine or NAC treatment would have prevented the obliteration of NO formation observed after NEM treatment alone. The increase in NO formation after treatment with these thiol compounds indicates that tissue thiols are somewhat limiting with respect to the biotransformation of GTN to NO in BPA in vitro, such that supplementation with L-cysteine or NAC was able to enhance NO formation. This interpretation differs from that of Boesgaard et al. (1993), who observed that supplementation of rats with oxothiazolidine, which increases intracellular glutathione and cysteine concentrations, did not potentiate GTN-induced vasodilation. According to the NO prodrug hypothesis, if NO formation from GTN is enhanced, it follows that a corresponding shift in the dose-response curve for GTN should be observed, which was not seen. The data obtained with D-cysteine in the protection protocol were also inconsistent with the NO prodrug hypothesis, in that D-cysteine was unable...
to prevent the shift in the GTN dose-response curve induced by NEM treatment but was able to maintain NO formation at a level similar to that of the controls.

In the case of the reversal protocol, a difference between the experimentally obtained data and those predicted by the NO produg hypothesis was observed. Treatment with L-cysteine or NAC did not restore the original relaxation of the BPA, but the formation of NO exceeded that of the controls. For D-cysteine treatment, there was no reversal of the NEM effect even though there was recovery of the ability to form NO from GTN. The expectation based on the dose-response curves for GTN-induced relaxation was that treatment with thiol agents after NEM should have yielded partial recovery of the ability to form NO, but full or greater recovery was not predicted.

The finding that the inhibitory effects of NEM on GTN-induced vasorelaxation in this study were blocked, but not reversed, by treatment with the L-enantiomers of thiol-containing compounds indicates that this inhibition is not caused solely by the alkylation of free thiol groups, thereby preventing a nonenzymatic interaction of these thiol groups with GTN to produce NO. If a direct nonenzymatic reaction between a thiol compound and GTN were responsible for NO formation in vascular tissue, supplementation with thiol compounds should have reversed the effect of NEM. Incubation of tissue in the presence of NAC or L-cysteine has been shown to increase intracellular cysteine and glutathione concentrations (Greutter and Lemke, 1985; Anderson and Meister, 1987). If the mechanism by which NEM attenuated GTN-induced relaxation involved decreases in the concentrations of free thiols in the BPA tissue, one would have expected incubation with NAC or L-cysteine after exposure to NEM to replenish these concentrations, thus allowing the BPA rings to relax to the same extent as the controls. Such a reversal of the inhibitory effects of NEM was not observed, suggesting that NEM irreversibly inhibits a portion of the GTN biotransformation pathway in BPA.

One proposed mechanism for the metabolic activation of GTN involves enzymatic conversion of the organic nitrates to an S-nitrosothiol in the presence of cysteine and stimulation of soluble guanylyl cyclase by this NO adduct, either directly or indirectly via the release of NO (Kurz et al., 1991). Indeed, an enzyme that appears to be involved in the biotransformation of GTN has been isolated from the cell membranes of VSM cells (Seth and Fung, 1993). This enzyme is believed to contain a free thiol group that is required for catalytic activity. Its activity was augmented by the addition of thiol-containing compounds, such as L-cysteine and NAC, and inhibited by NEM and other reagents known to bind free thiol groups. Inhibition of this or a similar enzyme could explain the irreversible attenuation of GTN-induced vasorelaxation after NEM treatment. More importantly, a role for an S-nitrosothiol considerably changes the expectations for a good correlation between NO formation from organic nitrates and the ability of the nitrates to bring about vascular relaxation. In conclusion, the results of this study indicate that direct formation of NO from organic nitrate vasodilators may not be the route of their metabolic activation; the data confirm a role for sulfhydryl groups in the metabolism of GTN and in its ability to induce VSM relaxation.

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