Effect of In Vitro Organic Nitrate Tolerance on Relaxation, Cyclic GMP Accumulation, and Guanylate Cyclase Activation By Glyceryl Trinitrate and the Enantiomers of Isoidide Dinitrate

Brian M. Bennett, Henning Schröder, L. Douglas Hayward, Scott A. Waldman, and Ferid Murad

Previously, it was shown that the D enantiomer of isoidide dinitrate was 10-fold more potent than the L enantiomer and 10-fold less potent than glyceryl trinitrate for stimulating cyclic GMP accumulation and relaxation of isolated rat aorta. In the present study, these organic nitrates were tested for their ability to induce tolerance to organic nitrate-induced relaxation, cyclic GMP accumulation, and guanylate cyclase activation in rat aorta in vitro. To compensate for the differences in vasodilator potency, tolerance was induced by incubating isolated rat aorta with concentrations of organic nitrates 1,000-fold greater than the EC50 for relaxation. Under these conditions, the EC50 for relaxation was increased significantly for each organic nitrate and to a similar degree on subsequent reexposure. These data suggest that the potential for inducing in vitro tolerance to relaxation was the same for the three organic nitrates tested. When activation of soluble guanylate cyclase by these compounds was assessed, the enantiomers of isoidide dinitrate were equipotent, but less potent than glyceryl trinitrate, suggesting that the site of enantioselectivity is not guanylate cyclase itself. In blood vessels made tolerant to organic nitrates by pretreatment with glyceryl trinitrate, vasodilator activity, cyclic GMP accumulation, and guanylate cyclase activation were attenuated on reexposure to each organic nitrate. In addition, differences in the potency of the three organic nitrates and the enantioselectivity of isoidide dinitrate for relaxation were abolished in tolerant tissue, whereas the potency difference between glyceryl trinitrate and isoidide dinitrate for activation of guanylate cyclase was unchanged. The results suggest that there are at least two sites at which organic nitrates act: guanylate cyclase and an unidentified site that allows for enantioselectivity of vasodilation and cyclic GMP accumulation. Furthermore, when blood vessels are made tolerant to organic nitrates, the enantioselectivity and the differences in potency of the organic nitrates for relaxation are lost, and activation of guanylate cyclase is decreased. (Circulation Research 1988;63:693–701)

Organic nitrates and other nitrovasodilators are thought to induce smooth muscle relaxation by activation of soluble guanylate cyclase (GTP pyrophosphate-lyase, EC 4.6.1.2), resulting in the intracellular accumulation of cyclic GMP.1–3 Although the precise molecular mechanism by which organic nitrates activate guanylate cyclase is unknown, it may involve the release or formation of nitric oxide, which then acts as the proximal moiety for enzyme activation.1–4 Recently the biotransformation of the organic nitrate glyceryl trinitrate (GTN) to glyceryl dinitrate was demonstrated to occur concurrently with relaxation of vascular smooth muscle induced by GTN.5 Also, biotransformation of GTN was attenuated in tissues.

From the Departments of Medicine and Pharmacology, Stanford University School of Medicine and Veterans Administration Medical Center, Palo Alto, California, and the Department of Chemistry (L.D.H.), University of British Columbia, Vancouver, British Columbia, Canada.

Supported in part by grants from the National Institutes of Health (AM-30787, HL-28474), the Veterans Administration, and the Council for Tobacco Research, U.S.A., Inc. B.M.B. is supported by a fellowship from the Medical Research Council of Canada.

Address for correspondence: Dr. Brian M. Bennett, Department of Pharmacology and Toxicology, Queen's University, Kingston, Ontario, K7L 3N6, Canada.

Address for reprints: Dr. Ferid Murad, Department of Medicine and Pharmacology (111), Stanford University of Medicine and Veterans Administration Medical Center, 3801 Miranda Avenue, Palo Alto, CA 94304.

Received June 11, 1987; accepted April 25, 1988.
made tolerant to the relaxant effect of GTN. Furthermore, in GTN-tolerant vascular tissues, accumulation of cyclic GMP and activation of guanylate cyclase by organic nitrates was attenuated. Thus, there appears to be a close association between the mechanism underlying organic nitrate action, tolerance, and biotransformation.

The dinitrate esters of 1,4:3,6-dianhydrohexitol exist in three stereoisomeric forms and differ in the spatial orientation of the nitrate ester groups (Figure 1). For isoidide dinitrate (IIDN), both nitrate groups are in the exo position. Isomannide dinitrate (IMDN) contains two endo nitrate groups, whereas isosorbitide dinitrate (ISDN) has one exo and one endo nitrate group. Studies comparing the relative vasodilator activity of the three isomers demonstrated an order of potency of IIDN > ISDN > IMDN. Presumably this difference in potency is related to the configuration of the nitrate ester groups since the three isomers are otherwise identical. In addition to the positional isomerism discussed above, each of these compounds is optically active. Only one enantiomer of each has been studied experimentally or clinically, since optical activity is retained after synthesis from the naturally occurring sugar alcohols, D-sorbitol, D-mannitol, and L-iditol. Recently we synthesized for the first time the D enantiomer of L-IIDN also was synthesized using D-mannitol as the starting material (U.S. and Canadian patent applications filed). The D-IIDN had an infrared spectrum and melting point matching that of L-IIDN and had a specific rotation, [α]D 25° of -97.6° compared with +98.2° for L-IIDN. Stock solutions of organic nitrates were prepared by extraction of organic nitrate-lactose powder (GTN, 10% wt/wt; D-IIDN and L-IIDN, 50% wt/wt) with ethanol. Further dilutions were made with Krebs' solution. Organic nitrate concentrations were determined by modification of the spectrophotometric method of Dean and Baum, in that reagent volumes were reduced by a factor of 20 and a KNO3 standard curve was used rather than a single KNO3 concentration. All other chemicals were of reagent grade and were obtained from a variety of sources.

Development of tolerance to the vasodilator effect of D- or L-IIDN has not been examined, and another objective was to compare the potential of D- and L-IIDN with that of GTN for inducing in vitro tolerance.

Materials and Methods

Drugs and Solutions

Krebs' solution was composed of the following (mM): NaCl 118, KCl 4.74, MgSO4 1.18, KH2PO4 1.18, CaCl2 2.5, NaHCO3 24.9, and glucose 10. The solution was aerated with 95% O2-5% CO2 and maintained at 37°C. GTN was obtained from ICI Americas, Wilmington, Delaware. L-IIDN was synthesized from D-mannitol as described previously. The D-IIDN had an infrared spectrum and melting point matching that of L-IIDN and had a specific rotation, [α]D 25°, of -97.6° compared with +98.2° for L-IIDN. Stock solutions of organic nitrates were prepared by extraction of organic nitrate-lactose powder (GTN, 10% wt/wt; D-IIDN and L-IIDN, 50% wt/wt) with ethanol. Further dilutions were made with Krebs' solution. Organic nitrate concentrations were determined by modification of the spectrophotometric method of Dean and Baum, in that reagent volumes were reduced by a factor of 20 and a KNO3 standard curve was used rather than a single KNO3 concentration. All other chemicals were of reagent grade and were obtained from a variety of sources.

Relaxation Studies

Male Sprague-Dawley rats (200-250 g) were decapitated and the thoracic aorta removed and cut into helical strips (approximately 2 mm x 1.5 cm, three strips per aorta). The strips were suspended in individual tissue baths containing 15 ml Krebs' solution, and isometric responses were recorded using force displacement transducers (FTO3C, Grass Instruments, Quincy, Massachusetts) coupled to a Grass Model 7 polygraph. The strips were maintained at a resting tension of 0.4 g for the 1-hour equilibration period, during which the Krebs' solution was changed every 15 minutes. Tissues were contracted submaximally with 0.3 μM norepinephrine, and after 10 minutes cumulative concentration-response curves for GTN, D-IIDN, or L-IIDN were obtained. The strips were washed for 30 minutes and organic nitrate tolerance was induced by one of two protocols. For "high-dose" organic nitrate tolerance, all strips were incubated with 0.5 mM GTN for 1 hour. The tissues were washed for 1 hour, contracted with 0.3 μM norepinephrine, and cumulative concentration-response curves were repeated for GTN, D-IIDN, or L-IIDN. For "low-dose" organic nitrate tolerance, the EC50 value for relaxation by the three organic nitrates in the first concentration-response...
Cyclic GMP Measurements

Four aortic strips were prepared from each rat and were incubated individually in flasks. After equilibration, two strips were incubated with 0.5 mM GTN for 1 hour and the other two strips were incubated with the appropriate volume of ethanol (<0.2%) in Krebs' solution. Tissues were washed for 1 hour, exposed to 0.3 μM norepinephrine for 10 minutes, and then to the organic nitrates at the concentrations and for the times indicated. Tissues were frozen between liquid-nitrogen precooled clamps and cyclic GMP levels were assayed as described previously. Briefly, frozen tissues were homogenized in 6% trichloroacetic acid and then centrifuged. Supernatant fractions were extracted with diethyl ether, acetylated, and cyclic GMP quantified by radioimmunoassay. Protein was determined by the method of Lowry et al using bovine serum albumin as the standard. In previous studies from this laboratory, both basal cyclic GMP levels and elevated cyclic GMP levels induced by various agents were similar when tissues were mounted under tension in tissue baths or placed in flasks. 

Guanylate Cyclase Activity

Aortas from three rats were pooled for determination of guanylate cyclase activity. Each aorta was cut open longitudinally and then divided in half. One segment was incubated with 0.5 mM GTN for 1 hour and the other served as control and was incubated with the appropriate volume of ethanol (<0.2%) in Krebs' solution. The segments were washed for 1 hour and then homogenized in ice-cold buffer containing 50 mM Tris-HCl (pH 7.6), 0.5 mM dithiothreitol, 1.0 mM EDTA, and 0.25 M sucrose. Homogenates were centrifuged at 105,000 g for 1 hour and the resulting supernatant fraction was used immediately for guanylate cyclase determinations. Guanylate cyclase activity was assayed essentially as described. Incubations (100 μl for 10 minutes at 37°C) contained 50 mM Tris-HCl (pH 7.6), 0.5 mM isobutylmethylxanthine, a GTP regenerating system consisting of 3.5 mM creatine phosphate and 6 μg creatine phosphokinase (190 units/mg protein), 1 mM L-cysteine, 5-6 μg supernatant protein, and other agents where indicated. Reactions were initiated by the addition of substrate (4 mM MgCl2, 1 mM GTP) and terminated by the addition of 0.9 ml of 50 mM sodium acetate (pH 4.0), followed by heating at 90°C for 3 minutes. Aliquots were assayed for cyclic GMP by radioimmunoassay.

Data from relaxation experiments and tissue cyclic GMP determinations are expressed as mean ± SD. Data from guanylate cyclase experiments are expressed as mean ± SEM. EC50 values were determined graphically from each concentration-response curve. Differences were analyzed by the appropriate statistical test as indicated; p<0.05 was considered statistically significant.

Results

High-Dose Organic Nitrate Tolerance

In agreement with an earlier study, GTN was approximately 10-fold and 100-fold more potent than D-IIDN and L-IIDN, respectively, for relaxation of isolated rat aortic strips (Figure 2). Induction of tolerance in vitro by exposure to 0.5 mM GTN resulted in a rightward shift of the concentration-response curve for each of the three organic nitrates tested. However, the difference in potency between the three organic nitrates, and the enantiomeric difference for relaxation by D- and L-IIDN was lost in tolerant tissues (Figure 2). The effect of high-dose GTN treatment on cyclic GMP accumulation was tested (Table 1). Although basal cyclic GMP levels were unaffected by GTN pretreatment to induce tolerance, there was a marked attenuation of cyclic GMP accumulation after expo-
TABLE 1. Effect of High-dose Glyceryl Trinitrate Pretreatment on Cyclic GMP Accumulation and Relaxation by Organic Nitrates

<table>
<thead>
<tr>
<th>Drug and treatment</th>
<th>Cyclic GMP (pmol/mg protein)*</th>
<th>Relaxation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal</td>
<td>Drug</td>
</tr>
<tr>
<td>Glyceryl trinitrate (1 μM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.20±0.01</td>
<td>2.20±0.20†</td>
</tr>
<tr>
<td>Tolerant</td>
<td>0.19±0.04</td>
<td>0.37±0.08</td>
</tr>
<tr>
<td>D-isoidide dinitrate (10 μM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.24±0.05</td>
<td>0.72±0.23†</td>
</tr>
<tr>
<td>Tolerant</td>
<td>0.21±0.04</td>
<td>0.21±0.02</td>
</tr>
<tr>
<td>L-isoidide dinitrate (10 μM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.28±0.05</td>
<td>0.60±0.13†</td>
</tr>
<tr>
<td>Tolerant</td>
<td>0.20±0.02</td>
<td>0.25±0.02</td>
</tr>
</tbody>
</table>

Cyclic GMP measurements were made at 0.5 minutes for glyceryl trinitrate and at 2 minutes for D- and L-isoidide dinitrate, time points at which cyclic GMP accumulation was maximal. Cyclic GMP values are mean±SD of three experiments. Relaxation data are taken from Figure 2, and values are mean±SD of four experiments.

*Data analyzed by one-way analysis of variance. Newman-Keuls test was used to determine which comparisons were statistically significant.
†Statistically significant from all other groups (p<0.01). All other comparisons were not statistically significant (p>0.05).
‡Statistically significant from control (p<0.01; Student’s t test for paired data).

Low-Dose Organic Nitrate Tolerance

The protocol for low-dose organic nitrate tolerance was developed to compare the ability of various organic nitrates with different potencies to induce tolerance. Since the shift in sensitivity was not expected to be very great using this protocol, it was important to control for possible time-dependent changes in the sensitivity of the preparation. As seen in Figure 3, pretreatment of the tissues with diluent and buffer alone resulted in only a slight shift of the concentration-response curves to the right. The EC50 values for relaxation before and after such treatment were not significantly different (p>0.05, Student’s t test for paired data). In contrast, treatment of blood vessels with any of the three organic nitrates resulted in a significant increase in the EC50 value for relaxation (Figure 4). The EC50 value for relaxation was shifted by a factor of 3.1-fold, 5.4-fold, and 6.1-fold for GTN, D-IIDN, and L-IIDN, respectively. However, these values are not significantly different when compared with each other (p>0.05, one-way analysis of variance).

Guanylate Cyclase Activity

Concentration-response curves for the activation of soluble guanylate cyclase prepared from nontolerant and high-dose GTN-tolerant rat aorta are shown in Figure 5. Concentration-dependent increases in enzyme activity were observed in both GTN-tolerant and control preparations in response to the organic nitrates and to sodium nitroprusside (SNP). Basal enzyme activity in control and GTN-tolerant preparations was not different (76±8 and 66±5 pmol cyclic GMP/mg protein/min, respectively; p>0.05, Student’s t test for paired data). The EC50 values for enzyme activation in nontolerant preparations were SNP, 3.2±0.3 μM; GTN, 38±9 μM; D-IIDN, 0.22±0.01 mM; and L-IIDN, 0.22±0.03 mM. These values were not significantly different when compared with each other (p>0.05, one-way analysis of variance). In contrast, treatment of blood vessels with any of the three organic nitrates resulted in a significant increase in the EC50 value for relaxation (Figure 4). The EC50 value for relaxation was shifted by a factor of 3.1-fold, 5.4-fold, and 6.1-fold for GTN, D-IIDN, and L-IIDN, respectively. However, these values are not significantly different when compared with each other (p>0.05, one-way analysis of variance).
Figure 4. Effect of "low-dose" organic nitrate treatment on concentration-response curves for glyceryl trinitrate (GTN), D-isoidide dinitrate (D-IIDN), and L-isoidide dinitrate (L-IIDN). Solid lines, concentration-response curves before organic nitrate treatment. EC50 value for relaxation from each preparation was calculated and each strip was incubated for 1 hour with concentration of organic nitrate that was 1,000-fold greater than the EC50. Tissues were washed for 1 hour and concentration-response curves repeated (dashed lines). Data points are mean±SD from four experiments. EC50 value for relaxation was significantly increased for each organic nitrate after treatment (p<0.01, Student's t test for paired data).

Discussion

There appear to be at least two processes associated with the activation of guanylate cyclase and cyclic GMP accumulation induced by organic nitrates: biotransformation of organic nitrates and direct activation of guanylate cyclase. It was hypothesized by Needleman and Johnson that organic nitrates interact with a specific SH group in the "organic nitrate receptor" and as a consequence of this interaction, relaxation ensues accompanied by denitration of the organic nitrate and oxidation of the SH group to the disulfide form. Others have suggested that activation of guanylate cyclase by organic nitrates involves release or formation of nitric oxide or S-nitrosothiols, which then act as the proximal moieties for enzyme activation. In both cases, biotransformation of organic nitrates is a prerequisite for activation of guanylate cyclase.

Previous studies have demonstrated that in GTN-tolerant vascular tissue, there is decreased biotransformation of GTN and decreased activation of soluble guanylate cyclase, suggesting that both of these sites are affected by pretreatment of tissues with GTN. Insight into the relative contribution of these two mechanisms on the diminished vasodilator response to GTN has been obtained in experiments that examined cross-tolerance between GTN and the nitrovasodilator SNP. Concentration-response curves for relaxation induced by GTN in GTN-tolerant blood vessels were shifted to the right up to 750-fold compared with nontolerant tissues, while those for SNP were shifted no more than threefold. Consistent with the different effect of these agents on relaxation in GTN-tolerant aorta, cyclic GMP accumulation induced by GTN was markedly attenuated in GTN-tolerant tissue, whereas cyclic GMP accumulation induced by SNP was only slightly decreased. This contrasts with results obtained in this study (Figure 5) and in a previous study in which activation by SNP and GTN of nitrovasodilators in GTN-pretreated tissues was significantly less than control at most drug concentrations tested (Figure 5). At drug concentrations eliciting maximal activation, enzyme activity was decreased by approximately 75% in GTN-tolerant preparations.

These results suggest that a stable alteration of the enzyme occurred that survived homogenization of the tissue and centrifugation. An alternative explanation for the diminished guanylate cyclase activity in GTN-tolerant tissues is that factors inhibitory to guanylate cyclase may be present in different amounts in extracts from tolerant and nontolerant tissues. However, in a previous study from this laboratory, guanylate cyclase activity from GTN-tolerant and nontolerant rat aorta was studied in both supernatant fractions and supernatant from which guanylate cyclase was purified by immunoprecipitation with a monoclonal antibody specific for guanylate cyclase. In GTN-pretreated tissues, enzyme activation by GTN, SNP, and nitric oxide was attenuated to a similar degree when either crude enzyme or immunoprecipitated enzyme was used. Since immunoprecipitation would be expected to remove any inhibitory factors if present, it is unlikely that such interference could explain the decrease in guanylate cyclase activity in tolerant tissues.
soluble guanylate cyclase obtained from GTN-tolerant rat aorta was inhibited to a similar degree. These data suggest that, although there is desensitization of the enzyme to activation, there is sufficient enzyme activity remaining to permit SNP-induced cyclic GMP accumulation and relaxation to be near to control values. This implies that alteration of the metabolic activation of organic nitrates is a more important determinant for organic nitrate-induced tolerance than desensitization of guanylate cyclase to activation per se, and that the pathways leading to the formation of active species are different for organic nitrates than for SNP.

Central to the above discussion is the question of the degree of guanylate cyclase activation required to produce sufficient cyclic GMP for relaxation. Evaluation of the literature is complicated by differences in experimental protocol with respect to the vascular preparation, the nature and concentration of the contractile agent, and the time of exposure to the relaxant compound. In experiments using rat or rabbit aorta, contracted submaximally with phenylephrine or norepinephrine and exposed to GTN for periods that elicited maximal cyclic GMP accumulation (30 seconds for rat aorta, 1.5–2 minutes for rabbit aorta), maximal or near maximal relaxation by GTN (0.1–1 μM) was associated with threefold to sevenfold increases in cyclic GMP levels.12,15,26,27 In another study in which aorta was contracted submaximally with norepinephrine and exposed to 0.1 μM SNP, near maximal relaxation occurred with less than a twofold increase in cyclic GMP levels.11 With higher concentrations of GTN or SNP, elevations of cyclic GMP of up to 300-fold have been reported,7,11,12 indicating a large reserve capacity for enzyme activation. With lower concentrations of GTN or SNP, significant relaxation has been associated with statistically insignificant changes in cyclic GMP levels,8,15,26 suggesting that vascular smooth muscle is quite sensitive to small alterations in cyclic GMP. Indeed, changes in cyclic GMP-dependent protein phosphorylation occur at concentrations of SNP (5 nM) at which changes in tissue cyclic GMP levels would be difficult to discern.28 To return to the question of whether there is sufficient enzyme activity remaining in GTN-tolerant tissues to allow for SNP-induced cyclic GMP accumulation and relaxation, the above examples would suggest an affirmative answer because 1) there appears to be a large reserve capacity for enzyme activation, 2) modest increases in cyclic GMP levels can result in relaxation, and 3) approximately 25% of guanylate cyclase activity remains in GTN-tolerant tissues (Figure 5).

Although it is possible that the modest degree of cross-tolerance to SNP in GTN-pretreated tissues may be due to desensitization of guanylate cyclase by GTN, other sites (e.g., a site of GTN biotransformation to an active species) must be affected by GTN-pretreatment because much larger decreases in relaxation and cyclic GMP accumulation occur on subsequent reexposure to GTN than to SNP.

Although D-IIDN was about 10-fold more potent than L-IIDN for vascular smooth muscle relaxation and cyclic GMP accumulation,13 the results of the present study demonstrate clearly that the two isomers were equipotent and were equally efficacious for activation of soluble guanylate cyclase (Figure 5). In addition, the concentrations of organic nitrates that induce relaxation and cyclic GMP accumulation in intact tissues are approximately three orders of magnitude less than those that activate soluble guanylate cyclase in broken cell preparations. This would suggest that the mechanism of activation of guanylate cyclase by organic nitrates in intact tissues is different than that in broken cell preparations. In this regard, it has been demonstrated that in broken cell preparations, activation of soluble guanylate cyclase by organic nitrates requires the addition of cysteine, N-acetylcysteine, or thiosalicylic acid to the incubation medium,29,30 but there is no thiol requirement for organic nitrate-induced cyclic GMP accumulation in intact tissue. It has been demonstrated
Recently, it was shown that cysteine reacts nonenzymatically with GTN, resulting in the liberation of nitric oxide, which then activates the enzyme. The concentration of nitric oxide released from the reaction of millimolar concentrations of GTN with cysteine is in the high nanomolar range, and is in agreement with the concentrations of nitric oxide required for enzyme activation. Thus, the high concentrations of organic nitrates required to activate guanylate cyclase in broken cell preparations can be explained by the rather inefficient formation of nitric oxide from the reaction of organic nitrates with cysteine. This process, or some other form of metabolic activation, is presumably much more efficient in intact cells, and therefore enzyme activation and subsequent relaxation occur at much lower concentrations of organic nitrates. It is of interest that the biotransformation of GTN during incubation with broken cell preparations from aortic tissue is exceedingly slow (3–4% in 8 hours) as compared with that obtained when intact aorta is used (8.5% in 2 minutes). Thus, the addition of thiols to broken cell preparations probably substitutes for some kind of metabolic activation process in intact tissues that is destroyed on homogenization of the tissue. Furthermore, the absence of enantioselectivity of IIDN for activation of guanylate cyclase in broken cell preparations would argue against a nonenzymatic reaction of organic nitrates with cysteine as the mechanism of enzyme activation in intact tissues.

Although it has been demonstrated that the biotransformation of GTN is attenuated in GTN-tolerant tissues, we wondered whether the enantioselectivity of IIDN for relaxation and cyclic GMP accumulation might be altered in tolerant tissues and whether this might reflect enantioselectivity in the biotransformation reaction. Induction of high-dose GTN tolerance resulted in rightward shifts of the concentration-response curves of approximately 5,000-fold, 400-fold, and 40-fold for GTN, D-IIDN, and L-IIDN, respectively (Figure 2). Furthermore, the enantioselectivity of IIDN and the potency difference between the three organic nitrates for relaxation seen in nontolerant aorta was absent in tolerant tissue. In fact, although D-IIDN was of intermediate potency in nontolerant aorta, it was the least potent organic nitrate in tolerant tissue, and was significantly less efficacious than GTN and L-IIDN. Cyclic GMP accumulation was examined at drug concentrations that elicited maximal relaxation in nontolerant tissue (GTN, 1 μM; D- and L-IIDN, 10 μM). At these concentrations, cyclic GMP accumulation was increased significantly in nontolerant tissues, but not in GTN-tolerant tissues. In addition, cyclic GMP accumulation induced by 100 μM or 1 mM D-IIDN in GTN-tolerant tissues was significantly less than that induced by the same concentrations of L-IIDN (data not shown). In contrast, D-IIDN was more potent than the L-isoemer for cyclic GMP accumulation in nontolerant blood vessels.

When activation of soluble guanylate cyclase was examined, there was a marked decrease in efficacy of the organic nitrates and of SNP for activation of enzyme from GTN-tolerant tissue, indicating that a stable alteration of the enzyme had occurred that survived homogenization of the tissue and centrifugation, as described previously. However, in contrast to the data from the relaxation experiments, the EC50 for enzyme activation and the order of potency of GTN, D-IIDN, and L-IIDN was the same in enzyme preparations from tolerant or nontolerant tissues (Figure 5). Again, this illustrates that activation of guanylate cyclase by organic nitrates is different in intact tissues and broken cell preparations. Together these data suggest that the site that determines the vasodilator potency of organic nitrates and enantioselectivity of organic nitrate action is lost when tissues are made tolerant to organic nitrates. Since decreased biotransformation of organic nitrates appears to be an important consequence of organic nitrate-induced tolerance, it is proposed that the site that determines enantioselectivity is a site of biotransformation.

Although the glutathione S-transferases catalyze the denitration of organic nitrates in the liver, these enzymes have not been isolated from vascular smooth muscle. It is of interest, however, that glutathione S-transferases from liver and lung exhibit enantioselectivity for the conjugation of glutathione with polycyclic aromatic hydrocarbons and other compounds. Biotransformation of GTN can also occur by interaction with hemoglobin and myoglobin, and there is regioselectivity in the denitration reaction in that there is a preferential formation of the 1,2-dinitrate metabolite over the 1,3-dinitrate metabolite. This regioselectivity for denitration also occurs in rabbit aorta, bovine pulmonary artery and vein, and in several cultured cell lines. It is therefore conceivable that vascular hemoproteins may participate in the biotransformation of organic nitrates and possibly enantioselective biotransformation. A third candidate is guanylate cyclase itself. Guanylate cyclase appears to contain a heme prosthetic group that can participate in enzyme activation. Isoidide dinitrate could interact in an enantioselective manner with this heme group or with another part of the molecule. However, if this does occur in situ, tissue homogenization results in the loss of this property since the two isomers are equipotent for activation of guanylate cyclase in cell-free preparations. Since enantioselectivity of IIDN action is affected by GTN tolerance, identification of the site responsible for enantioselectivity and an understanding of its properties may suggest ways in which tolerance to organic nitrates could be prevented or reversed.

Tolerance to the vasodilator effect of IIDN has not been examined, and one of our objectives was to assess the relative potential of GTN, D-IIDN, and
L-IIDN for inducing tolerance. The degree of in vitro tolerance induced by organic nitrates will depend on dose, potency, time of exposure, and length of time allowed for recovery. In the present experiments, a protocol was developed to compensate for the differences in vasodilator potency of the three organic nitrates, and to compensate for inter-animal variation in sensitivity to a particular organic nitrate. Tolerance induced by this protocol resulted in a significant shift to the right of concentration-response curves for the three organic nitrates tested (Figure 4). However, in each case the EC$_{50}$ for relaxation was increased to a similar degree, indicating that the potential for inducing in vitro tolerance was the same for each of the agents tested. Whether L-IIDN would be a useful therapeutic agent for the prophylaxis or treatment of angina pectoris awaits the characterization of its hemodynamic and pharmacokinetic properties. The results of the present study suggest that tolerance induced by L-IIDN was no greater than that induced by GTN.

Recent findings of Ahlner et al$^{44,45}$ are of interest in the context of the present study. They describe a biphasic concentration-response curve for GTN in bovine mesenteric artery and suggest the existence of high- and low-affinity components for GTN-induced relaxation, both of which are mediated by cyclic GMP.$^{44}$ Furthermore, exposure of tissues to low GTN concentration (0.1 nM) for long time periods (8 hours) resulted in selective attenuation of the high affinity component, whereas exposure to high GTN concentration (0.1 mM) affected relaxation at all GTN concentrations.$^{45}$ Consistent with these observations, relaxation reached a plateau at 70–85% relaxation with GTN concentration in the micromolar range (Figure 4), and although not shown, 100% relaxation could be obtained if GTN concentration was increased to the millimolar range.

The low-dose protocol for tolerance induction used in the present study resulted in rightward shifts in the concentration-response curves, but enantioselectivity and the potency difference between the organic nitrates was retained. On the other hand, high-dose GTN tolerance resulted in larger shifts in the concentration-response curves and a loss of enantioselectivity and the potency difference between the organic nitrates. Based on these findings, we propose that the high-affinity component for organic nitrate-induced relaxation is a site of biotransformation to an active species, which then activates guanylate cyclase. Organic nitrate action at this site is partially attenuated with low-dose organic nitrate pretreatment and completely lost with high-dose GTN-pretreatment. Consistent with the high concentration of organic nitrates needed for guanylate cyclase activation in cell-free preparations, and also the requirement for cysteine, the low affinity component for organic nitrate-induced relaxation may be the result of the nonenzymatic reaction of organic nitrates with cysteine leading to the generation of nitric oxide and subsequent activation of guanylate cyclase.

In summary, our results suggest that there are two sites at which organic nitrates act: guanylate cyclase and an unidentified site that determines the vasodilator potency and enantioselectivity of organic nitrate action and that is lost when tissues are made tolerant to organic nitrates. That the enantioselective site is a site of biotransformation is attractive since biotransformation of organic nitrates is related to relaxation, and in tolerant tissues both relaxation and biotransformation are attenuated and the enantioselectivity of organic nitrate action is lost.

Acknowledgments

The authors thank Beverly Lyons and Janet LeSarge for their assistance in the preparation of this manuscript.

References


37. Watabe T, Hirasuka A, Tsurumori T: Enantioselectivity in glutathione conjugation of 1,2-epoxy-1,2,3,4-tetrahydronaphthalene by hepatic glutathione S-transferase. Biochem Biophys Res Commun 1985;130:65–70.


42. Bennett BM, Leitman RL, Schroeder H, Murad F: Relationship between biotransformation of glyceryl trinitrate (GTN) and cyclic GMP accumulation in various cell lines (abstract). FASEB J 1988;2:A1066.


KEY WORDS: glycercyl trinitrate • isoidide dinitrate • cyclic GMP • guanylate cyclase • vascular smooth muscle.

Bennett et al. Tolerance, Cyclic GMP, and Organic Nitrates 701
Effect of in vitro organic nitrate tolerance on relaxation, cyclic GMP accumulation, and guanylate cyclase activation by glyceryl trinitrate and the enantiomers of isoidide dinitrate.
B M Bennett, H Schröder, L D Hayward, S A Waldman and F Murad

doi: 10.1161/01.RES.63.4.693

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1988 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/63/4/693

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation Research_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation Research_ is online at:
http://circres.ahajournals.org/subscriptions/