Effects of the Flavoprotein Inhibitor, Diphenyleneiodonium Sulfate, on Ex Vivo Organic Nitrate Tolerance in the Rat

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ABSTRACT

The flavoprotein inhibitor, diphenyleneiodonium (DPI), inhibits the action of glyceryl trinitrate (GTN) and the $\alpha$-enantiomer of isoidide dinitrate (IIDN), but not the $\beta$-enantiomer (L-IIDN), in isolated rat aorta via inhibition of the bioactivation of these prodrugs. Paradoxically, a vascular NAD(P)H oxidase, which also is inhibited by DPI, has been proposed to generate superoxide that quenches nitric oxide (NO) produced during GTN biotransformation, and increased oxidase levels are proposed to contribute to the phenomenon of organic nitrate tolerance. We examined the effect of DPI on isolated rat aorta using an in vivo model of organic nitrate tolerance. The $EC_{50}$ values for GTN-$\alpha$, L-IIDN-$\beta$, and L-IIDN-induced relaxation of aorta from GTN-tolerant rats were increased 4.5- to 7.5-fold. Treatment of blood vessels with DPI ($0.3 \mu M$) increased the $EC_{50}$ values for GTN and $\alpha$-IIDN by the same magnitude in control and tolerant aortae, a result that would not be predicted if DPI and GTN tolerance affected common targets. The expression of NADPH-cytochrome P450 reductase (CPR) in vivo and in vitro was assessed by NADPH-dependent cytochrome c reductase activity of aortic microsomes, immunoblotting, and Northern analysis. By all three determinants, CPR expression was unchanged in aorta from GTN-tolerant rats. Superoxide dismutase-inhibitable NADPH-dependent cytochrome c reductase activity (a measure of superoxide generation) of tolerant rat aortic microsomes was not different from that of controls. Superoxide dismutase-inhibitable NAD(P)H-dependent cytochrome c reductase activity was detected only in microsomes from tolerant animals. DPI caused a modest increase in the sensitivity for relaxation by the NO donor DEA NONOate to an equal extent in tolerant and nontolerant tissues, whereas the superoxide scavenger, 4,5-dihydroxy-1,3-benzene disulfonic acid (Tiron), had no effect on the sensitivity for relaxation by GTN. These results would not be expected if tolerance-induced increases in superoxide were a causative factor for the reduced relaxation response in tolerance. We conclude that neither reduced flavoprotein-dependent metabolic activation of organic nitrates, such as that mediated by CPR, nor increased superoxide due to increased NAD(P)H oxidase activity can account for the development of in vivo tolerance to GTN.

Glyceryl trinitrate (GTN) has been used therapeutically for the treatment of angina pectoris and congestive heart failure for more than a century. After continuous dosing regimens with organic nitrates, the beneficial hemodynamic and anti-ischemic effects are diminished by the development of tolerance. The specific mechanism(s) leading to the development of tolerance to organic nitrates remains poorly understood, but the causes are generally considered to be multifactorial.

One mechanism of tolerance suggests an impaired bio-transformation of GTN to an active intermediate [presumably nitric oxide (NO) or a closely related compound] (Brien et al., 1988). It is known that vascular enzyme systems, such as the glutathione (GSH) S-transferases (Tsuchida et al., 1990; Nigam et al., 1996) and cytochromes P450 (McDonald and Bennett, 1993), are involved in the vascular biotransformation of GTN. However, evidence for the formation of NO from GTN at pharmacologically relevant drug concentrations is lacking. Another proposed tolerance mechanism is that superoxide, generated by a vascular NAD(P)H oxidase, is increased during tolerance, resulting in a quenching of NO generated during organic nitrate biotransformation (Münzel et al., 1995).

Studies from our laboratory have shown that the flavoprotein inhibitor, diphenyleneiodonium sulfate (DPI), targets enzymes that are involved in the biotransformation of organic nitrates and that are involved in the vasodilator response to organic nitrates. McGuire et al. (1994) reported that DPI caused parallel rightward shifts of the concentration-response curves for GTN-induced relaxation that corre-

ABBRVIATIONS: 1,2-GDN, glyceryl-1,2-dinitrate; CPR, NADPH-cytochrome P450 reductase; DPI, diphenyleneiodonium sulfate; GSH, glutathione; GTN, glyceryl trinitrate; IIDN, isoidide dinitrate; NO, nitric oxide; SOD, superoxide dismutase; DEA NONOate, 1,1-diethyl-2-hydroxy-2-nitrosohydrazine, sodium salt; GSNO, S-nitrosothiol; ACh, acetylcholine.
lated to decreased biotransformation [specifically, decreased glyceryl-1,2-dinitrate (1,2-GDN) formation] and decreased GTN-induced cGMP accumulation in isolated rat aorta. More recently, Ratz et al. (1999) showed that DPI abolished the enantioselective differences for relaxation, cGMP accumulation, and biotransformation between the δ- and ε-enantiomer of isoidide dinitrate (IIDN) and L-IIDN in rat aorta. It was also demonstrated that DPI inhibited the hemodynamic response to GTN and L-IIDN in an in vivo rat model and altered their pharmacokinetic properties (McGuire et al., 1998; Ratz et al., 1999). Using an in vivo rat model of GTN tolerance, we have shown that in aorta from these animals there is a rightward shift in the concentration response for GTN-induced relaxation that correlates with a decrease in cGMP accumulation and a decrease in GTN biotransformation (J.D.R., A. B. Fraser, and B.M.B., unpublished observations).

Although all the possible flavoprotein targets of DPI in blood vessels have not been determined, several vascular and nonvascular flavin-containing enzymes that are inhibited by DPI have been identified, viz., NADPH-cytochrome P450 reductase (CPR) (McGuire et al., 1998), an NAD(P)H oxidase (Griendling et al., 1994), xanthine oxidase/dehydrogenase (O'Donnell et al., 1993), mitochondrial NADH-ubiquinone oxidoreductase (Majander et al., 1994), and endothelial NO synthase (Stuehr et al., 1991). Of particular relevance are CPR and the NAD(P)H oxidase because altered activity of these enzymes may be involved in organic nitrate tolerance either through changes in GTN bioactivation or in superoxide-generating activities, respectively.

Based on the similar effects of DPI and in vivo GTN tolerance on the functional and biochemical alterations in responsiveness to organic nitrates, we hypothesized that a common target may be affected by the two treatment regimens. If this were the case, one would predict that the inhibitory effects of DPI on organic nitrate action would be attenuated in tolerant tissues. Therefore, we compared the effects of DPI on the relaxation response to organic nitrates; the NO donor, 1,1-diethyl-2-hydroxy-2-nitrosohydrazine, sodium salt (DEA NONOate); and the S-nitrosothiol, S-nitroso glutathione (GSNO), in tolerant and nontolerant tissues. We also tested whether in vivo GTN tolerance altered the expression or activity of rat aortic CPR or altered aortic microsomal superoxide generation.

Materials and Methods

Drugs and Solutions. Krebs' solution was composed of the following: 118 mM NaCl, 4.74 mM KCl, 1.18 mM MgSO4, 1.18 mM KH2PO4, 2.5 mM CaCl2, 24.9 mM NaHCO3, and 10 mM glucose. The solution was aerated with 95% O2, 5% CO2 and maintained at 37°C. DPI was purchased from Color Your Enzyme (Kingston, Ontario, Canada). Stock solutions of IIDN were prepared by extraction of organic nitrate-lactose powder (50% w/w) with ethanol. Further dilutions were made with the appropriate buffer solution. The concentrations of GTN, 1,2-GDN, glyceryl-1,3-dinitrate, and IIDN in stock solutions were determined by a modification of the spectrophotometric method of Dean and Baum (1975) as described previously (Bennett et al., 1988). GSNO was prepared by combining 100 mM NaN3O and 100 mM GSH in aqueous solution and adjusting the pH to 2.0 with 0.1 M HCl. After a 5 min incubation period at room temperature, the solution was adjusted to pH 7.0 with 0.1 M NaOH and stored on ice. The absorbance at 394 nm was measured, and the concentration of GSNO was determined using an extinction coefficient of 767 M-1 cm-1.

GTN-induced cGMP accumulation in isolated rat aorta was determined by a modification of the spectrophotometric method of Dean and Baum (1975) as described previously (Bennett et al., 1988). GSNO was prepared by combining 100 mM NaN3O and 100 mM GSH in aqueous solution and adjusting the pH to 2.0 with 0.1 M HCl. After a 5 min incubation period at room temperature, the solution was adjusted to pH 7.0 with 0.1 M NaOH and stored on ice. The absorbance at 394 nm was measured, and the concentration of GSNO was determined using an extinction coefficient of 767 M-1 cm-1.

Materials and Methods

Induction of GTN Tolerance In Vivo. Male Sprague-Dawley rats (250–300g; Charles River, St. Constant, Quebec, Canada) were exposed to a continuous source of GTN via the subdermal implantation of transdermal GTN patches (tolerant) or drug-free patches (control) for 48 h. Rats were anesthetized with halothane before surgery. A small area was shaved in the upper dorsal region, and the site was disinfected with 2.5% iodine tincture. A transverse incision (2 cm) was made, and then the skin was separated from the underlying fascia by blunt dissection. Two transdermal patches were inserted (nondelivery surfaces back-to-back) into the subdermal opening. The site was sutured closed and disinfected again with iodine tincture. After 24 h, the site was reopened, and the patches were replaced. Forty-eight hours after the initial implantation of patches, the animals were sacrificed, and the thoracic aorta was removed for use in the following experimental protocols.

Relaxation Studies. Thoracic aortic strips were prepared from control and GTN-tolerant rats as described (Stewart et al., 1989). Two or three aortic strip preparations were obtained from each animal. In some experiments using GTN, the endothelium was removed by gentle scraping with a scalpel blade. Endothelium removal was confirmed by the absence of a relaxation response to acetylcholine (ACh; 10 nM–1 μM). Tissues were contracted maximally with 10 μM phenylephrine to ensure the viability of the preparation. After a 30 min washout period, the tissues were relaxed submaximally with 0.1 μM phenylephrine. Once the induced tone had stabilized, tissues were exposed to diluent (control), 0.3 μM DPI (DPI treated), Tiron (10 mM), or 100 μM allopurinol, and 15 min later, cumulative concentration-response curves for GTN (0.1 nM–0.1 mM), b-IIDN (1 nM–0.1 mM), or L-IIDN (10 nM–0.1 mM) were obtained. In another series of experiments, aortic strips from control or GTN-tolerant animals were exposed to diluent (control) or 0.3 μM DPI, and concentration-response curves were obtained for DEA NONOate (0.1 nM–1 μM) or GSNO (1 nM–3 μM). In a third strip from each animal, a GTN concentration-response curve was obtained to allow comparison of the degree of GTN tolerance achieved.

Biotransformation of GTN by Purified Xanthine Oxidase or Rat Aortic Supernatant. Purified xanthine oxidase (25 μg/ml) was incubated for 30 min with 1 μM GTN and 1 mM xanthine in 50 mM potassium phosphate buffer (pH 7.7) containing 0.1 mM EDTA. Incubations were performed in an Instrumentation Laboratory (Milan, Italy) 237 tonometer at 37°C under anaerobic (humidified N2 gas at a flow rate of 250 ml/min) conditions. To examine the inhibition of GTN biotransformation by DPI, samples were pretreated with 10 μM DPI and 0.1 mM xanthine for 15 min and then incubated with GTN. Values for nonenzymatic denitration were subtracted from those obtained in the presence of enzyme. In other experiments, the
105,000xg supernatant fraction of rat aorta was prepared as described (Nigam et al., 1996), and aliquots (200 μg/ml protein) were incubated anaerobically with 1 μM GTN and 1 mM xanthine or 1 mM NADH. The GTN metabolites, 1,2-GDN and glyceryl-1,3-dinitrate, were quantitated by megabore capillary column gas-liquid chromatography as described previously (McDonald and Bennett, 1990).

**NADPH- and NADH-Dependent Cytochrome c Reductase Activity and Superoxide Generation by Aortic Microsomes and Purified Xanthine Oxidase.** For the determination of NADPH-dependent cytochrome c reductase activity, aortic microsomes were prepared as described previously (McGuire et al., 1998) using three to six rats per group. For each harvested aorta, the endothelial layer was removed by gentle scraping with a scalpel blade before homogenization. For the determination of NADH-dependent cytochrome c reductase activity, aortic microsomes were prepared from individual rats. In this case, however, we chose to prepare microsomes from endothelium-intact aortas. The NADPH-dependent or NADH-dependent cytochrome c reductase activity of aortic microsomes was determined by spectrophotometric measurement of the reduction of bovine heart cytochrome c (Yasukochi and Masters, 1976). Also, NADPH- or NADH-dependent aortic microsomal superoxide production was determined by the SOD-inhibited rate of cytochrome c reduction using this assay. Samples (1 ml) for microsomal assays consisted of aortic microsomes [5–8 μg protein/ml (NADPH-dependent) or 30–50 μg protein/ml (NADH-dependent)] in 50 mM potassium phosphate buffer (pH 7.7) containing 0.1 mM EDTA, 36 μM cytochrome c, and, either diluent or SOD (300 U/ml). Reactions were initiated by the addition of either NADPH (100 μM) or NADH (100 μM), and the A550 followed for 5 min at 25°C using a Cary WinUV 100 spectrophotometer (Varian, Mississauga, Ontario, Canada). Reaction rates of cytochrome c reductase activities were calculated using the extinction coefficient for cytochrome c of 0.021 μM cm⁻¹.

**Northern Analysis.** Total tissue RNA was isolated from control or GTN-tolerant, de-endothelialized aortas using Qiagen (Mississauga, Ontario, Canada) RNeasy spin columns according to the manufacturer’s recommendations. Yields of 15 to 25 μg of total RNA per aorta were common. Total RNA (15 μg) was resolved on 1% agarose gels containing 1.1% formaldehyde and was transferred to nylon membranes (Hybond N⁺; Amersham, Piscataway, NJ) by overnight capillary blotting. Membranes were prehybridized in Quickhyb solution (Stratagene, La Jolla, CA) for 30 min at 68°C and then hybridized for 2 h at 68°C with cDNA probes that were 32P-radiolabeled with [32P]dCTP (6000 Ci/mmol) by random priming. Membranes were visualized by autoradiography, and quantitation was performed by phosphorimager analysis using a Molecular Dynamics (Sunnyvale, CA) Storm phosphorimager. The 952-base pair CPR cDNA probe was generated by an reverse transcriptase-polymerase chain reaction procedure using cultured rat aortic smooth muscle cells as the source of RNA. After quantitation of CPR mRNA, membranes were stripped and reprobed with a G3PDH probe (Clontech, Palo Alto, CA) to correct for variations in RNA loading.

**Immunoblot Analysis of NADPH-Cytochrome P450 Reductase Content in Aortic Microsomes.** Aortic microsomal proteins were separated on 10% gels by SDS-polyacrylamide gel electrophoresis and transferred electrophoretically to polyvinylidene difluoride membranes. Blots were then probed with a specific antibody to CPR that was kindly provided by Dr. B. S. S. Masters (University of Texas Health Science Center, San Antonio, Texas), and immunoreactive bands were visualized by enhanced chemiluminescence. The content of CPR in microsomes from control or GTN-tolerant rats was quantitated as follows. For each quantitation reported, additional lanes on the gels contained known amounts of CPR, and these were used to generate standard curves for band intensity and content. Quantitation of CPR content in aortic microsomes was performed using Corel Photo-Paint version 7.0 software (Corel Corp., Ottawa, Ontario, Canada) after the film was scanned using a Hewlett-Packard desktop scanner (Palo Alto, CA). A standard curve for enhanced chemiluminescence signal (arbitrary units) versus known amounts of enzyme was determined for the blot shown (correlation coefficients were $R^2 = 0.99$ and $R^2 = 0.98$ for two different sets of exposures).

**Data Analysis.** All data are presented as the mean ± S.D. EC₅₀ values were determined from each concentration-response curve by interpolation. Data from experiments designed with single treatments were analyzed by Student’s t test for paired or unpaired data as indicated. Data from all experiments with more than one treatment group were analyzed by a one-way ANOVA and Newman-Keuls post hoc test for multiple comparisons. The assumption of homogeneity of variance was tested in all cases using Bartlett’s test. Due to inhomogeneity of variance, statistical analysis for the relaxation experiments was performed using logarithmically transformed data. A P value of .05 or less was considered statistically significant.

**Results**

**Relaxation Studies.** The following results are summarized by comparing the rightward shift from the control EC₅₀ value due to the following treatments: GTN patch-induced tolerance, 0.3 μM DPI treatment, or the combination of both GTN patch-induced tolerance and DPI treatment. The EC₅₀ value for GTN-induced relaxation was shifted to the right 50-fold due to tolerance, 4.8-fold due to DPI treatment, and 50-fold due to both tolerance and DPI treatment together (Fig. 1). The EC₅₀ value for D-IIDN-induced relaxation was shifted to the right 7.6-fold due to tolerance, 6.2-fold due to DPI treatment, and 6-fold due to DPI treatment and 44-fold due to the combined effects of tolerance and DPI treatment (Fig. 2). For L-IIDN, GTN tolerance caused a similar increase in the EC₅₀ value for relaxation (4.5-fold). However, treatment with DPI alone increased (by 2.0-fold), rather than decreased, the sensitivity for L-IIDN-induced relaxation (although this increase in sensitivity was not statistically significant), and this occurred to an equal extent in both GTN-tolerant and nontolerant tissues (Fig. 3). Previous results have shown that DPI only has an inhibitory effect on the D-enantiomer of IIDN (Ratz et al., 1999), a result that appears to hold true in tolerant tissues as well. These results indicate that the inhibitory effect of DPI on the relaxation response to GTN and D-IIDN was at least additive.

Fig. 1. Effect of GTN tolerance. 0.3 μM DPI, or both on GTN-induced relaxation of isolated rat aorta. The EC₅₀ values for GTN-induced relaxation were as follows: control, 3.5 ± 2.6 nM; tolerant, 23 ± 12 nM; control + DPI, 22 ± 16 nM; tolerant + DPI, 180 ± 57 nM. Each value represents the mean ± S.D. (n = 11–13). All EC₅₀ values were significantly different to each other except tolerant versus control + DPI (P < .001, one-way ANOVA).
The shift in the EC$_{50}$ value for relaxation in tolerant tissues made tolerant to GTN, one would expect a less than additive effect of GTN tolerance, 0.3 μM DPI, or both on D-IIDN-induced relaxation of isolated rat aorta. The EC$_{50}$ values for D-IIDN-induced relaxation were as follows: control, 0.19 ± 0.16 μM; control + DPI, 0.22 ± 0.21 μM; tolerant + DPI, 1.6 ± 1.5 μM. Each value represents the mean ± S.D. (n = 8). All EC$_{50}$ values were significantly different to each other except tolerant versus control + DPI (P < .001, one-way ANOVA).

Fig. 2. Effect of GTN tolerance, 0.3 μM DPI, or both on D-IIDN-induced relaxation of isolated rat aorta. The EC$_{50}$ values for D-IIDN-induced relaxation were as follows: control, 37 ± 18 nM; tolerant, 0.28 ± 0.16 μM; control + DPI, 0.090 ± 0.052 μM; tolerant + DPI, 0.45 ± 0.16 μM. Each value represents the mean ± S.D. (n = 8). All EC$_{50}$ values were significantly different to each other except tolerant versus control + DPI (P < .001, one-way ANOVA).

Fig. 3. Effect of GTN tolerance, 0.3 μM DPI, or both on L-IIDN-induced relaxation of isolated rat aorta. The EC$_{50}$ values for L-IIDN-induced relaxation were as follows: control, 0.14 ± 0.14 μM; tolerant, 0.86 ± 0.26 μM; control + DPI, 0.090 ± 0.052 μM; tolerant + DPI, 0.45 ± 0.16 μM. Each value represents the mean ± S.D. (n = 6). All EC$_{50}$ values were significantly different to each other except control versus control + DPI and tolerant versus tolerant + DPI (P < .01, one-way ANOVA).

The relaxation response to GSNO was not altered in GTN-tolerant tissues or in tissues treated with DPI (Fig. 5b), whereas the EC$_{50}$ value for GTN-induced relaxation was increased by 7.1-fold in this series of experiments. Treatment of tissues with Tiron did not alter the EC$_{50}$ value for GTN-induced relaxation in either control or GTN-tolerant aortae (Fig. 6), indicating that scavenging of superoxide does not increase the sensitivity for relaxation by GTN. In other experiments, treatment of tissues with 100 μM allopurinol did not affect relaxation by GTN, D-IIDN, or L-IIDN (data not shown). These results suggest that xanthine oxidase activity does not play a significant role in the relaxation response to these organic nitrates.

**Effect of DPI on Biotransformation of GTN by Purified Xanthine Oxidase or Rat Aortic Supernatant.** Incubation of GTN (1 μM) and xanthine (1 mM) with purified xanthine oxidase (25 μg/ml) resulted in the biotransformation of GTN under anaerobic but not aerobic conditions. The rate of total GDN formation was 125 ± 5.4 pmol/min/mg protein (n = 5). After treatment of xanthine oxidase with 10 μM DPI, total GDN formation was significantly inhibited (99% or 1.6 ± 1.2 pmol/min/mg protein, n = 5). Under anaerobic conditions, biotransformation of GTN did not occur in the 105,000g supernatant of rat aorta supplemented with 1 mM xanthine or 1 mM NADH.
Expression of NADPH-Cytochrome P450 Reductase and Microsomal NAD(P)H Oxidase Activities during In Vivo GTN Tolerance.

Figure 7 illustrates the detection of mRNA and protein for CPR in aorta samples from control and GTN-tolerant rats. The size of the mRNA corresponded with that expected of CPR (2.4 kilobase pairs) as determined by its migration relative to RNA size standards (not shown). There were no differences for mRNA expression of CPR in blood vessels between control and GTN-tolerant rats. The mean ratio of aorta RNA for CPR to G3PDH (CPR:G3PDH) for control rats \((n = 4)\) was 2.0 \(\pm\) 0.6, and the ratio for GTN-tolerant rats \((n = 4)\) was 2.3 \(\pm\) 0.8 \((P > .05, \text{Student's } t \text{-test for unpaired data})\). There were no differences in CPR protein expression or enzyme activity in aortic microsomes from control compared with tolerant animals (Fig. 7 and Table 1). For the preparations shown in Fig. 7, the content of CPR in aorta from control rats \((n = 3)\) was 0.71 \(\pm\) 0.05 ng CPR/\(\mu\)g microsomal protein, and the content for GTN-tolerant rats \((n = 3)\) was 0.62 \(\pm\) 0.04 ng CPR/\(\mu\)g microsomal protein \((P = .1073, \text{Student's } t \text{-test for unpaired data})\). Approximately 25% of the NADPH-dependent cytochrome c reductase activity was inhibited by SOD, indicating the presence of NADPH oxidase activity in rat aortic microsomes (Table 1). However, the relative amount of NADPH-dependent superoxide formation was unaltered in microsomes from GTN-tolerant animals. The NADH-dependent cytochrome c reductase activity in aortic microsomes was approximately 30-fold greater than NADPH-dependent activity. In control microsomes, only a very small, nonsignificant amount of activity could be inhibited by SOD, indicating that NADH oxidase activity is very low in this tissue. In microsomes from GTN-tolerant animals, however, there was a small, but significant, decrease in NADH-dependent cytochrome c reduc-

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**Fig. 5.** Effect of GTN tolerance, 0.3 \(\mu\)M DPI, or both on DEA NONOate- (a) or GSNO-induced (b) relaxation of isolated rat aorta. The \(EC_{50}\) values for DEA NONOate-induced relaxation were as follows: control, 9.2 \(\pm\) 4.8 nM; tolerant, 22 \(\pm\) 10 nM; control + DPI, 5.1 \(\pm\) 2.1 nM; tolerant + DPI, 14 \(\pm\) 5.5 nM. Each value represents the mean \(\pm\) S.D. \((n = 6)\). EC\(_{50}\) values were significantly different for control versus tolerant, control + DPI versus tolerant + DPI, and control + DPI versus tolerant \((P < .01, \text{one-way ANOVA})\). The \(EC_{50}\) values for GSNO-induced relaxation were as follows: control, 26 \(\pm\) 19 nM; tolerant, 39 \(\pm\) 17 nM; control + DPI, 25 \(\pm\) 9 nM; tolerant + DPI, 52 \(\pm\) 5 nM. Each value represents the mean \(\pm\) S.D. \((n = 4)\). None of the EC\(_{50}\) values were significantly different to each other \((P > .05, \text{one-way ANOVA})\).

**Fig. 6.** Effect of GTN tolerance, 10 mM Tiron, or both on GTN-induced relaxation of isolated rat aorta. The \(EC_{50}\) values for GTN-induced relaxation were as follows: control, 3.5 \(\pm\) 2.5 nM; tolerant, 11 \(\pm\) 2.8 nM; control + Tiron, 4.0 \(\pm\) 1.7 nM; tolerant + Tiron, 12 \(\pm\) 6.4 nM. Each value represents the mean \(\pm\) S.D. \((n = 5)\). EC\(_{50}\) values were significantly different to each other for all comparisons except control versus control + Tiron and tolerant versus tolerant + Tiron \((P < .05, \text{one-way ANOVA})\).

**Fig. 7.** Effect of GTN tolerance on the levels of vascular CPR mRNA and protein. a, Northern analysis of rat aortic RNA (15 \(\mu\)g of total RNA per lane) from three control and two GTN-tolerant animals. The signal ratio of CPR:G3PDH was unchanged in aortae from GTN-tolerant animals. b, Immunoblot analysis of CPR (5 \(\mu\)g of microsomal protein per lane) in aorta from three control and three GTN-tolerant animals. CPR protein levels were unchanged in aortae from GTN-tolerant animals.
tase activity in the presence of SOD, suggesting the presence of an NADH oxidase in tolerant vascular tissue. The SOD-inhibited activity accounted for approximately 5% of the total; the remainder likely reflects NADH-cytochrome b₅ reductase activity.

Discussion

The results from this study suggest that, at least in the rat, DPI does not target an enzyme system or process that is responsible for the rightward shift in the concentration-response curve for GTN or D-IIDN seen after the development of GTN tolerance in vivo. This is consistent with the data indicating that the mRNA, protein levels, and activity of aortic CPR and rat aortic microsomal NADPH-oxidase activity, two identified targets of DPI in isolated rat aorta, were unaffected during in vivo GTN tolerance. In a previous study (McGuire et al., 1998), DPI was shown to be an effective inhibitor of anaerobic GTN biotransformation by purified CPR and to inhibit aortic CPR activity and aortic CPR-dependent GTN biotransformation. In this study, DPI inhibited the xanthine-dependent GTN biotransformation by xanthine oxidase. These results are consistent with the ability of DPI to inhibit the activities of these two flavoproteins.

Previous data obtained in the rat suggested that DPI was targeting an enzyme that may be involved in the development of GTN tolerance (McGuire et al., 1994, 1998; Ratz et al., 1999). Based on relaxation and biochemical studies using aortae from nontolerant rats, DPI treatment resulted in a rightward shift in the concentration-response curve to GTN and D-IIDN that correlated with decreases in organic nitrate sensitivity for L-IIDN-induced relaxation in both control and GTN-tolerant tissues. These results are consistent with the ability of DPI to inhibit the activities of these two flavoproteins.

### Table 1

<table>
<thead>
<tr>
<th>Aortic Microsome Preparation</th>
<th>NADPH-Cytochrome c Reductase Activitya</th>
<th>NADH-Cytochrome c Reductase Activityb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>16.3 ± 2.8</td>
<td>491 ± 47</td>
</tr>
<tr>
<td>Control + SOD</td>
<td>12.0 ± 2.2</td>
<td>475 ± 49</td>
</tr>
<tr>
<td>Tolerant</td>
<td>16.8 ± 3.5</td>
<td>450 ± 47</td>
</tr>
<tr>
<td>Tolerant + SOD</td>
<td>11.0 ± 1.4</td>
<td>425 ± 40</td>
</tr>
</tbody>
</table>

a Each value is the mean ± S.D. of two to four measurements for n preparations using five to six rats per preparation.

b Each value is the mean ± 5% of measurements in microsomes from five rats.

P < .05 (control versus control + SOD and tolerant versus tolerant + SOD, Student’s t test for paired data).

Further studies have provided good evidence that CPR is a target for inhibition by DPI. Incubation of aortic microsomes with 125I-labeled DPI resulted in the labeling of proteins (79 and 50 kDa) that would be consistent with the binding of DPI to both CPR and cytochromes P450 (McGuire and Bennett, 1996). Furthermore, DPI was shown to inhibit aortic CPR activity and to inhibit NADPH-dependent GTN biotransformation in aortic microsomes (McGuire et al., 1998).

In this study, the shift in the concentration-response curve for GTN- or D-IIDN-induced relaxation was similar in tolerant tissues and tissues treated with DPI. However, the shift was at least additive when these treatments were combined. This would not have been expected if common targets were affected by GTN tolerance and by DPI. Furthermore, we were able to dissociate the effects of tolerance and of DPI using L-IIDN. In this case, the concentration-response curves for L-IIDN-induced relaxation were shifted to the right in aortae from tolerant animals, whereas DPI slightly increased the sensitivity for L-IIDN-induced relaxation in both control and GTN-tolerant tissues.

Allopurinol, DPI, and SOD were all found to be effective inhibitors of purified xanthine oxidase activity (superoxide production) as measured by inhibition of cytochrome c reduction (data not shown). In previous studies, SOD has been used to enhance ACh-induced relaxation by preventing the destruction of NO by superoxide (Mian and Martin, 1995). Other studies have suggested that the superoxide that is generated from xanthine oxidase scavenges NO produced by endothelial NOS (Miyamoto et al., 1996; Ellis et al., 1998). Our findings would not be consistent with the hypothesis that DPI acts solely as an inhibitor of superoxide generation. According to this hypothesis, it would be expected that a potent inhibitor of xanthine oxidase such as DPI would enhance the relaxation response to organic nitrates via its inhibitory effect on xanthine oxidase-mediated superoxide production. The opposite effect was observed in our study where DPI shifted the concentration-response curve to the right for GTN and D-IIDN. Under anaerobic conditions only, purified xanthine oxidase biotransformed GTN to its dinitrate metabolites in a xanthine-dependent manner, and this biotransformation was almost completely inhibited by DPI. Recently, it has been shown that purified bovine xanthine oxidase catalyzes the reduction of GTN to NO under hypoxic conditions using NADH as the reducing substrate (Millar et al., 1998). To determine whether this cytosolic enzyme played a role in vascular organic nitrate biotransformation, GTN and added xanthine or NADH were incubated with the
105,000 g supernatant of rat aorta. Organic nitrate biotransformation was not observed under anaerobic conditions, and, therefore, it seems unlikely that the inhibitory actions of DPI could be due to inhibition of xanthine oxidase-mediated organic nitrate biotransformation activity. Combined with the lack of an effect of allopurinol on the functional relaxation response to all three organic nitrates used in this study, this would suggest that xanthine oxidase does not play a role in the biotransformation of organic nitrates that leads to relaxation in rat aortic tissue.

Results of a number of recent studies have provided evidence for the involvement of the renin-angiotensin system in the development of GTN tolerance in vivo (Kurz et al., 1999). The proposed mechanism involves activation of the renin-angiotensin system in response to chronic exposure to GTN, increased levels of circulating angiotensin II, and increased vascular superoxide production via a protein kinase C-mediated increase in the activity of a vascular NAD(P)H oxidase. The increased superoxide is thought to quench NO formed from nitrovasodilators, thus reducing the NO-mediated activation of guanylate cyclase and inhibiting vasodilation. These studies have been performed in a rabbit model of in vivo tolerance using transdermal GTN patches, a model very similar to our rat model of in vivo tolerance. These investigators found that the reduced vasodilator response to GTN in aortae from GTN-tolerant animals was associated with an increase in vascular superoxide production that could be inhibited with DPI (Münzel et al., 1995). The vasodilator response to ACh also was attenuated, and both responses could be restored by treatment with a membrane-permeable form of SOD. In addition, the vasodilator response to GTN in tolerant aorta was increased on removal of the endothelium (Münzel et al., 1995) or addition of the superoxide scavenger, Tiron (Münzel et al., 1999). In other studies, in vivo GTN tolerance was associated with an increase in plasma renin activity and vascular AT1 receptor mRNA levels (Kurz et al., 1999) and a decrease in the expression of vascular SOD (Münzel et al., 1999). Furthermore, cotreatment of rabbits with losartan during GTN tolerance induction resulted in normalization of vascular superoxide levels and of GTN-induced relaxation of aorta from GTN-tolerant animals. Our data using the rat model of in vivo GTN tolerance has produced conflicting results. We found that the relaxation response to GTN in aorta from GTN-tolerant animals was unchanged after endothelium removal (Fig. 4) and that ACh-induced relaxation was unaltered in GTN-tolerant tissues. This suggests that there may be a fundamental difference in the process of tolerance development between species with respect to the contribution of the endothelium.

In this study, NADPH oxidase activity, measured as SOD-inhibited cytochrome c reductase activity, accounted for approximately 25% of the total cytochrome c reductase activity of aortic microsomes with the remainder being CPR activity. Neither of these activities was altered in microsomes prepared from GTN-tolerant rats. The NADH oxidase activity of aortic microsomes was difficult to assess because of the high background of cytochrome c reduction by NADH-cytochrome b5 reductase present in the microsomal fraction. In microsomes from untreated animals, cytochrome c reductase activity was not significantly decreased in the presence of SOD. In microsomes from GTN-tolerant animals, the SOD-inhibited activity was 26.4 ± 10.6 nmol cytochrome c reduced/min/mg protein, which was approximately 5% of the total NADH-dependent cytochrome c reductase activity of the preparation. This is in contrast to NADH-dependent superoxide production reported in previous studies in rat and rabbit (Münzel et al., 1995, 1996; Rajagopalan et al., 1996; Kurz et al., 1999; Münzel et al., 1999) and in numerous other reports as well. For example, rat aortic microsomal NADH oxidase activity was reported to be approximately 600 nmol superoxide/min/mg protein in the study of Rajagopalan et al. (1996). In rabbit aortic microsomes, the NADH oxidase activity was approximately 250 nmol superoxide/min/mg protein, and this was increased to approximately 800 nmol superoxide/min/mg protein in microsomes from GTN-tolerant rabbits (Münzel et al., 1996).

The reasons for these discrepancies likely are related to differences in assay methodology. In this study, we used a standard and well-established assay for superoxide detection, viz., SOD-inhibited cytochrome c reductase activity (McCord and Fridovich, 1969). In the studies referenced above, 250 μM lucigenin was used in the lucigenin chemiluminescence assay for superoxide detection. The use of high concentrations of lucigenin has been shown in several systems to result in the generation of superoxide via the auto-oxidation of the lucigenin cation radical, thus resulting in overestimation of superoxide formation (Liochev and Fridovich, 1997; Vásquez-Vivar et al., 1997; Li et al., 1998; Skatchkov et al., 1999). In addition, lucigenin-dependent chemiluminescence signals are generated during reduction of lucigenin by microsomal NAD(P)H oxidoreductases (e.g., CPR and NADH-cytochrome b5 reductase) and subsequent decomposition catalyzed by the heme moiety of cytochrome P450 (Schechtman, 1999). Nevertheless, in contrast to this study in rat, GTN tolerance in the rabbit was associated with a marked increase in vascular superoxide production, again suggesting a fundamental difference in the process of tolerance development between the two species. The more relevant question is whether GTN tolerance is associated with increased superoxide production during sustained GTN treatment in humans. Although several studies have reported that antioxidants such as vitamins C and E can prevent tolerance development to GTN (Watanabe et al., 1997, 1998; Bassenge et al., 1998), only one clinical study has attempted to assess free radical production during sustained GTN treatment (Milone et al., 1999). As indices of oxidative stress, these authors measured plasma lipid peroxidation products, vitamin C, and 8-iso-prostaglandin F2α, and found that none of these were altered in GTN-tolerant subjects. Furthermore, administration of vitamin C to GTN-tolerant subjects did not restore the hemodynamic responses to GTN. The authors concluded that increased free radical production is not associated with GTN tolerance.

Because DPI and Tiron inhibit vascular superoxide production, one would predict that they would enhance GTN-induced vasodilation by preventing the superoxide-mediated destruction of GTN-derived "NO". However, Tiron had no effect on the EC50 value for GTN-induced relaxation in either tolerant or nontolerant tissues. The effects of DPI are complicated by its inhibitory action on CPR, which would serve to prevent the initial bioactivation of GTN, with the net effect of inhibition of GTN-induced vasodilation. Because l-arginine-induced relaxation and biotransformation is not inhibited by DPI (Ratz et al., 1999), it would appear that bioactivation of this organic nitrate is not mediated by a DPI-inhibited pro-
cess, and one would predict that a functional effect of DPI inhibition of NADP(H) oxidase could be observed with this organic nitrate. DPI did cause an increase in the sensitivity for l-IIIIND-induced relaxation (although this was not statistically significant), which would be consistent with a role for NADP(H) oxidase-derived superoxide in modifying the relaxation response. If the basis for the decreased relaxation response in tolerance was due to increased superoxide formation (NADH oxidase activity did increase as yet another factor), one would predict that inhibition of superoxide formation would have a greater effect in tolerant tissues and would normalize the sensitivity for relaxation. This was not the case because DPI increased the sensitivity for l-IIIIND-induced relaxation to a small but equal extent in both control and tolerant tissues, suggesting that factors other than enhanced superoxide formation are responsible for the decreased vasodilator response in tolerance. This was further reinforced by the inability of the superoxide scavenger, Tiron, to increase the sensitivity for GTN-induced relaxation in GTN-tolerant tissues.

To exclude the complications of metabolic activation of organic nitrates in this interpretation, we used the NO donor, DEANONOate, as a source of NO and assessed responses to DPI in tolerant tissues. In this case, there was partial cross-tolerance for relaxation by DEANONOate in GTN-tolerant tissues, but, like l-IIIIND, the slight increase in sensitivity for relaxation in DPI-treated tissues occurred to an equal extent in both tolerant and nontolerant blood vessels. Again, if there were functional consequences of increased superoxide production in tolerant tissues, one would expect a greater effect of DPI in these blood vessel preparations. Thus, although NADP(H) oxidase-derived superoxide may play a role in modifying the response to NO, we would contend that our data are not consistent with a GTN tolerance-induced increase in superoxide as a causative factor for the reduced relaxation response in tolerance. We also performed similar experiments with the S-nitrosothiol, GSNO. In this case, there was very little change in the sensitivity for relaxation in GTN-tolerant tissues, and relaxation responses were not altered by DPI. If the increase in sensitivity for relaxation by DEANONOate in DPI-treated tissues is due to superoxide, the lack of effect of DPI on the relaxation response to GSNO would be consistent with the much slower rate of reaction of superoxide with S-nitrosothiols compared with NO (Aleryani et al., 1998; Trujillo et al., 1998).

Taken together, it has been shown that although DPI can inhibit the pharmacological actions of organic nitrates (McGuire et al., 1994; Ratz et al., 1999) in a manner similar to that seen after the induction of GTN tolerance, in this study we showed that DPI does not appear to target proteins in common with those that may be affected during the development of GTN tolerance in vivo. Also, several flavoprotein-dependent activities that are inhibited by DPI and that have been considered putative mediators of tolerance via superoxide production do not appear to be involved in tolerance either. We suggest that other enzyme systems known to be involved in the biotransformation of organic nitrates, e.g., glutathione S-transferases (Tsuchida et al., 1990; Nigam et al., 1996) or others (Chung et al., 1992) or downstream components in the guanylate cyclase-GMP system, may be more likely candidates for modification during the development of GTN tolerance in vivo.


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