Inhibition of Phosphodiesterase 5 Selectively Reverses Nitrate Tolerance in the Venous Circulation

Jeffery D. MacPherson, Timothy D. Gillespie, Heather A. Dunkerley, Donald H. Maurice, and Brian M. Bennett

Department of Pharmacology and Toxicology, Faculty of Health Sciences, Queen's University, Kingston, Ontario, Canada

ABSTRACT

An important component of the antianginal efficacy of glyceryl trinitrate (GTN) is attributable to its selective venodilator effect, resulting in decreased cardiac preload and myocardial oxygen demand. Tolerance to nitrates occurs during chronic exposure, and the current study assessed whether this was due to increased phosphodiesterase (PDE) activity in the venous circulation. Tolerance was induced in rats by continuous exposure to 0.4 mg/h GTN for 48 h. Tension recordings of isolated femoral artery and vein indicated that tolerance was more pronounced in femoral vein. 4-[[3,4-(Methyleneedioxy)benzyl]amino]-6-chloroquinazoline (MBCQ), a selective PDE5 inhibitor, significantly decreased the EC50 values for GTN-induced relaxation in both tolerant and nontolerant tissues, but with the greatest relative shift occurring in tolerant veins. MBCQ also increased the vasodilator potency of 1,1-diethyl-2-hydroxy-2-nitrosohydrazine (DEA/NO), a nitric oxide donor; however, cross-tolerance between DEA/NO and GTN was not observed. A significant increase in cGMP PDE activity was observed in tolerant femoral vein, whereas PDE activity was unchanged in femoral artery. Conscious rats treated with hexamethonium (30 mg/kg) to induce ganglionic blockade exhibited blunted central venous pressure (CVP) and mean arterial pressure (MAP) responses to bolus i.v. doses of GTN in GTN-tolerant animals. The cGMP PDE inhibitor zaprinast (1 mg/kg) selectively reversed the blunted CVP response to GTN in tolerant animals but had no effect on the CVP response to GTN in nontolerant animals or on the MAP response in either group. These results suggest that increased PDE5 activity in the venous circulation contributes to the altered hemodynamic response to GTN following chronic GTN exposure.

Organic nitrates such as glyceryl trinitrate (GTN) have been used clinically since the 19th century for treatment of angina pectoris and congestive heart failure. A major clinical benefit of nitrates is attributed to their preferential venodilator effect, resulting in decreased venous return, decreased cardiac preload, and decreased myocardial oxygen demand. However, tolerance to the hemodynamic and antianginal actions of organic nitrates has been problematic with respect to their clinical effectiveness during long-term treatment. Mechanisms that have been proposed to contribute to nitrate tolerance include: intravascular volume expansion (Dupuis et al., 1990), neurohumoral counter-regulation (Parker et al., 1991), decreased biotransformation of nitrates to an active metabolite (Brien et al., 1986; Bennett et al., 1989; Slack et al., 1989; Sage et al., 2000; Chen et al., 2002), diminished activity of soluble guanylyl cyclase (sGC), or increased activity of phosphodiesterases (PDEs) (Axelsson and Andersson, 1983; Ahlner et al., 1986; Waldman et al., 1986; Kim et al., 2001), depletion of critical sulfhydryl groups (Needleman and Johnson, 1973), increased production of superoxide anion (Münzel et al., 1995b), decreased activity of vascular superoxide dismutase (Münzel et al., 1999), and increased vascular endothelin-1 production (Münzel et al., 1995a; Table 1). There is evidence to both support and refute many of these mechanisms, suggesting that the development of tolerance to the therapeutic effects of GTN is a complex and multifactorial process.

It is widely held that nitrate-induced vasodilation is mediated by activation of sGC and increased cGMP accumulation, resulting in activation of cGMP-dependent protein kinase (PK) G, and phosphorylation of cell proteins, leading to decreased intracellular Ca2+ and/or decreased Ca2+ sensitivity. An alternate or additional mechanism by which increases in cGMP may mediate relaxation is inhibition of the cAMP-hydrolyzing PDE, PDE3, resulting in increased cAMP levels. This work was supported by Grant T 5162 from the Heart and Stroke Foundation of Ontario.

Abbreviations: GTN, glyceryl trinitrate; sGC, soluble guanylyl cyclase; PDE, phosphodiesterase; PK, protein kinase; CVP, central venous pressure; MAP, mean arterial pressure; DEA/NO, 1,1-diethyl-2-hydroxy-2-nitrosohydrazine; MBCQ, 4-[[3,4-(methyleneedioxy)benzyl]amino]-6-chloroquinazoline; Ca/CAM, calcium/calmodulin; ANOVA, analysis of variance.

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and activation of cAMP-dependent PKA or PKG (Maurice and Haslam, 1990; Maurice et al., 1991). An increase in the catabolism of cGMP in vascular smooth muscle cells would be expected to reduce the relaxation response to nitrates and might contribute to nitrate tolerance. The enzymes responsible for cGMP catabolism are the cyclic nucleotide phosphodiesterases, which catalyze the hydrolysis of cGMP and cAMP to the corresponding 5′-nucleotide monophosphate. The cGMP-specific PDE5 and the calcium/calmodulin-dependent PDE1 are the PDE isoforms that mediate the hydrolysis of cGMP in vascular smooth muscle cells (Polson and Strada, 1996; Francis et al., 2001; Maurice et al., 2003), although the relative importance of each isoform varies with species and blood vessel type.

Several studies have provided evidence for a role of PDEs (e.g., PDE1A1 and PDE5) in nitrate tolerance (Axelsson and Andersson, 1983; Ahnler et al., 1986; Silver et al., 1991; Pagani et al., 1993; de Garavilla et al., 1996; Kim et al., 2001). Most studies investigating nitrate tolerance have used arterial tissue, mainly thoracic aorta tissue preparations, or have assessed changes in mean arterial pressure when this phenomenon has been studied in vivo. However, given the preferential action of nitrates on the venous circulation (Mackenzie and Parratt, 1977; Abrams, 1995; Parker and Parker, 1998) and certain studies suggesting selective GTN tolerance in the venous circulation (Stewart et al., 1986; Ohi et al., 1992; Bauer and Fung, 1996), we wished to investigate the role of PDE5 in nitrate tolerance in both the arterial and venous circulation. We used an in vivo GTN tolerance model and assessed vasodilator responses of isolated femoral artery and vein ex vivo and also assessed central venous pressure (CVP) and mean arterial pressure (MAP) responses in conscious unrestrained animals.

**Materials and Methods**

**Drugs and Solutions.** Transdermal GTN patches were obtained as Transderm-Nitrobrand (0.2 mg/h) from Novartis Pharmaceuticals (Dorval, QC, Canada). Drug-free patches were produced by soaking patches for at least 2 days in 95% ethanol (patches were allowed to air dry for 15 min prior to implantation). TRIDIL GTN injection USP (5 mg/ml) in ethanol/propylene glycol/water (1:1:1.33) was obtained from Sabex Inc. (Boucherville, QC, Canada), and further dilutions were made using Krebs’ solution. 1,1-Diethyl-2-hydroxy-2-nitrosohydrzone (DEA/NO) was obtained from Calbiochem (La Jolla, CA). 4-[(3,4-Methylenedioxy)benzyl]aminio]-6-chloroquinazoline (MBCQ) and zaprinast were purchased from Tocris (Ballwin, MO). For i.v. injection, zaprinast was solubilized in 0.5 N NaOH and diluted 1:10 in sterile saline to a final concentration of 1 mg/ml. The anti-PDE5A antibody was a generous gift of Dr. S. S. Visweswariah (Indian Institute of Science, Bangalore, India).

**Induction of GTN Tolerance in Vivo.** All procedures for animal experimentation were undertaken in accordance with the principles and guidelines of the Canadian Council on Animal Care. GTN tolerance was induced by exposing rats (male Sprague-Dawley, 275–325 g) to a continuous source of GTN via the subdermal implantation of two 0.2 mg/h transdermal GTN patches (tolerant) or drug-free patches (control) for 48 h as described previously (Ratz et al., 2000a). Briefly, a 1-cm transverse incision was made, and the skin was separated from the underlying fascia by blunt dissection. Two transdermal patches were inserted back-to-back into the resulting subdermal space. The site was sutured closed and disinfected with 2.5% iodine. Patches were replaced after 24 h; 24 h later, animals were sacrificed, and the femoral arteries and veins were removed.

**In Vitro Relaxation Studies.** Isolated rings of femoral artery and vein from GTN-tolerant or control animals were prepared for isometric tension measurements and were equilibrated for 1 h at an optimal resting tension of 2.5 mN for femoral veins and 5 mN for femoral arteries. Two artery and two vein preparations were obtained from each animal. Tissues were pretreated with 1 μM MBCQ or vehicle (0.3% dimethyl sulfoxide) for 10 min, contracted submaximally with phenylephrine (1–5 μM), and after the induced tone had stabilized, cumulative concentration-response curves were obtained for GTN (0.1 nM–0.1 mM) or DEA/NO (0.1 nM–3 μM). Preliminary experiments established that the concentration of MBCQ used was the minimum concentration that produced a maximal leftward shift in the concentration-response curve for GTN.

**cGMP Phosphodiesterase Activity Assay.** Femoral arteries and veins were homogenized in lysis buffer consisting of 5 mM Tris-HCl, pH 7.4, 5 mM MgCl2, 5 mM benzamidine, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1% Triton X-100 and centrifuged at 3000 g for 3 min. Cyclic GMP PDE activity was assayed by a modification (Rose et al., 1997) of the method of Davis and Daly (1979) using 0.5 to 2 μg of homogenate protein and 1 μM [3H]-cyclic GMP (600–900 dpm/pmol) as substrate. Triplicate samples were incubated for 30 min at 37°C, and the reaction product ([3H]-5′-GMP), was purified by column chromatography and quantified by liquid scintillation counting. In some samples, cGMP PDE activity was assessed in the presence of 1 μM MBCQ or a combination of 800 μM Ca2+ and 4 μg/ml calmodulin (Ca/CAM). The cGMP PDE activity was expressed as picoatoms per minute per milligram of protein.

**Immunoblot Analysis.** Femoral arteries and veins, vena cava, and thoracic aorta from six to eight control or GTN-tolerant rats, in four separate experiments, were pooled and homogenized in 0.25 M sucrose, 50 mM Tris HCl, and 0.5 mM EDTA, pH 7.2, containing 0.1 mM phenylmethylsulfonyl fluoride and protease inhibitors (Roche Diagnostics, Mannheim, Germany) and centrifuged at 480 g. Proteins in the supernatant fraction were separated on 7.5% reducing gels and transferred electrophoretically to polyvinylidene difluoride membranes. Blots were then probed with a rabbit polyclonal antibody to rat PDE5A (1:5000 dilution), and immunoreactive bands were visualized by enhanced chemiluminescence.

**Hemodynamic Studies.** Male Sprague-Dawley rats (250–275 g) had catheters (30-gauge Teflon fused to 0.508-mm i.d. Tygon) inserted into the abdominal vena cava (one for recording CVP and another for drug administration) and the abdominal aorta (for recording MAP). The animals were administered buprenorphine (Buprenex, 0.03 mg/kg) for pre- and postsurgical analgesia as required and were anesthetized for surgery with a 1:2:1 mixture of Hypnorm, sterile water, and Versed (0.3 ml/kg). The catheters were externalized between the scapulae, sutured into position, and filled with a solution containing 200 IU/ml heparin and 0.2 ml/g dextrose to help maintain patency during the ensuing 24-h postsurgery recovery period. Animals then underwent the in vivo GTN tolerance protocol as described above. Continuous arterial and venous blood pressure...
measurements were obtained on conscious, unrestrained animals by
connecting the aortic and venous catheters to a Statham pressure
transducer (P23ID; Grass Instruments, Quincy, MA) coupled to a
MacLab (ADInstruments, Toronto, ON, Canada) data acquisition
system. The animals were allowed to adjust to the blood pressure
recording conditions for approximately 1 h to establish a stable
baseline pressure. In a first group of animals, bolus i.v. doses of 30,
100, 300, 1000, and 3000 μg/kg GTN were administered to GTN-
tolerant or control rats. After a 30-min washout period, 30 mg/kg
hexamethonium was administered to induce ganglionic blockade,
and the dose-response curve was repeated 20 min later. A second
group of GTN-tolerant or control rats were administered 30 mg/kg
hexamethonium, and 20 min later, a GTN dose-response curve was
obtained. After a 30-min washout period, animals were administered
1 mg/kg zaprinast or vehicle, and 5 min later, the GTN dose-response
curve was repeated.

Data Analysis. All data are presented as the mean ± S.E.M. In
isolated blood vessel studies, the EC50 values for relaxation were
determined from each concentration-response curve using a sigmoi-
dal concentration-response curve-fitting algorithm. 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 logistically trans-
formed data. In the hemodynamic studies, only the absolute changes
in MAP or CVP are shown, but similar results were obtained when
percent changes in MAP or CVP were analyzed. The -fold shifts in
EC50 values due to inhibitor treatment were calculated and com-
pared using Student’s t test for unpaired data. Other groups of data
were compared using the appropriate statistical test, as indicated. A
p value of 0.05 or less was considered statistically significant.

Results

In Vitro Relaxation Studies. The in vivo protocol for
GTN tolerance resulted in approximately a 2-fold increase in the
mean EC50 value for GTN-induced relaxation in isolated
femoral artery, whereas that for the isolated femoral vein
was increased approximately 4-fold (Fig. 1, A and B), sug-
4.0 nM; and tolerant versus tolerant
MBCQ were significantly different (p < 0.05, one-way analysis of vari-
ance (ANOVA) and Newman-Keuls post hoc test). The EC50 values
(mean ± S.D.) for GTN-induced relaxation of femoral vein were: control,
17 ± 5.3 nM; tolerant, 64 ± 27 nM; control + MBCQ, 8.5 ± 4.0 nM; and
tolerant + MBCQ, 23 ± 14 nM. The EC50 values for control versus
tolerant, control versus control + MBCQ, and tolerant versus tolerant +
MBCQ were significantly different (p < 0.05, one-way ANOVA and
Newman-Keuls post hoc test). Each value represents the mean ± S.E.M.
(n = 5–8).

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Fig. 1. Effect of MBCQ (1 μM) on GTN-induced relaxation in the isolated
femoral artery (A) and the isolated femoral vein (B) of GTN-tolerant and
nontolerant animals. The EC50 values (mean ± S.D.) for GTN-induced
relaxation of femoral artery were: control, 17 ± 7.8 nM; tolerant, 34 ± 13
nM; control + MBCQ, 6.2 ± 3.0 nM; and tolerant + MBCQ, 31 ± 17 nM.
The EC50 values for control versus tolerant and control versus control +
MBCQ were significantly different (p < 0.05, one-way analysis of vari-
ance (ANOVA) and Newman-Keuls post hoc test). The EC50 values
(mean ± S.D.) for GTN-induced relaxation of femoral vein were: control,
17 ± 5.3 nM; tolerant, 64 ± 27 nM; control + MBCQ, 8.5 ± 4.0 nM; and
tolerant + MBCQ, 23 ± 14 nM. The EC50 values for control versus
tolerant, control versus control + MBCQ, and tolerant versus tolerant +
MBCQ were significantly different (p < 0.05, one-way ANOVA and
Newman-Keuls post hoc test). Each value represents the mean ± S.E.M.
(n = 5–8).

Fig. 2. Shift in potency (EC50/EC50 + MBCQ) after incubation of MBCQ
(1 μM) with control and GTN-tolerant femoral artery and vein. Each
value represents the mean ± S.E.M. (n = 5–8). The means of the indi-
vidual -fold shifts in potency were compared using Student’s t test for
unpaired data. *, significant difference in shift in potency caused by
MBCQ between control and tolerant groups (p < 0.05).

also assessed (Fig. 3). A lack of cross-tolerance between GTN
and DEA/NO in GTN-tolerant tissues is evident since both
GTN-tolerant and nontolerant tissues were equally responsi-
ve to DEA/NO. For both the GTN-tolerant and nontolerant
artery and vein preparations, preincubation with MBCQ re-
sulted in approximately a 5-fold increase in sensitivity to
DEA/NO-induced relaxation.
cGMP Phosphodiesterase Activity. cGMP PDE activity was assessed in homogenates of rat femoral artery (Fig. 4A) and vein (Fig. 4B) from control and GTN-tolerant animals. Basal cGMP PDE activity in femoral arteries from control animals was approximately 3-fold higher than that in femoral veins. There was a significant increase in basal PDE activity in femoral veins from tolerant animals, whereas basal enzyme activity in femoral arteries was unchanged, indicating that GTN treatment resulted in a selective change in PDE activity in the venous circulation. The relative contribution of PDE5 and PDE1 to cGMP PDE activity was assessed using MBCQ and Ca/CAM. The addition of MBCQ to the samples resulted in an 80 to 85% inhibition of PDE activity, suggesting that most of the activity present in these blood vessels can be attributed to PDE5. This was further substantiated by the finding that the addition of Ca/CAM to the samples did not alter enzyme activity, suggesting minimal contribution of the calcium/calmodulin-dependent PDE1 isoforms to the overall cGMP PDE activity.

Effect of GTN Tolerance on PDE5 Protein Levels. An immunoblot-based approach was used to determine whether GTN treatment altered the expression of PDE5 in rat arteries and veins. Basal enzyme activity in femoral veins from tolerant animals, whereas basal enzyme activity in femoral arteries was unchanged, indicating that GTN treatment resulted in a selective change in PDE activity in the venous circulation. The relative contribution of PDE5 and PDE1 to cGMP PDE activity was assessed using MBCQ and Ca/CAM. The addition of MBCQ to the samples resulted in an 80 to 85% inhibition of PDE activity, suggesting that most of the activity present in these blood vessels can be attributed to PDE5. This was further substantiated by the finding that the addition of Ca/CAM to the samples did not alter enzyme activity, suggesting minimal contribution of the calcium/calmodulin-dependent PDE1 isoforms to the overall cGMP PDE activity.

Hemodynamic Studies. In intact, conscious animals, the changes in CVP in response to i.v. bolus injections of GTN were highly variable, and there was no clear dose-response relationship (Fig. 6A). To overcome this problem, we induced ganglionic blockade with hexamethonium (30 mg/kg) to inhibit the reflex increases in sympathetic discharge that would be expected in response to administration of a vasodilator. This resulted in a reproducible, dose-dependent lowering of CVP in response to GTN and revealed significant
differences in the venodilator effect of GTN in tolerant and nontolerant animals (Fig. 6C). With respect to the MAP-lowering effect of GTN, there were small but significant differences between tolerant and nontolerant animals (Fig. 6B), and these were much more pronounced after ganglionic blockade (Fig. 6D).

We then examined the effects of the relatively selective PDE5 inhibitor zaprinast on the vasodilator responses to GTN in GTN-tolerant or nontolerant animals pretreated with hexamethonium (Fig. 7). In the absence of zaprinast, the attenuated vasodilator response to GTN is again evident in GTN-treated animals in both the arterial and venous sides of the circulation (Fig. 7, A and B). A relatively low dose of zaprinast was used in these experiments (1 mg/kg i.v.), one that alone had no effect alone on either CVP or MAP. This dose of zaprinast did not potentiate the vasodilator effects of GTN in nontolerant animals (Fig. 7, C and D) and had had no effect on the GTN-induced decrease in MAP in GTN-tolerant animals.
animals (Fig. 7F). In contrast, zaprinast significantly increased the responsiveness of GTN-tolerant animals to the CVP-lowering effect of GTN (Fig. 7E) to the extent that they were not different from those observed in nontolerant animals. Thus, inhibition of PDE in vivo selectively reversed tolerance to the venodilator actions of GTN.

Discussion

The clinical efficacy of nitrates in the treatment of congestive cardiac failure and as antianginal agents is due in large part to their selective actions on the venous side of the circulation. In heart failure, the nitrate-induced decrease in ventricular end diastolic pressure improves endocardial perfusion, and in angina, decreased preload reduces myocardial oxygen demand. Of the many mechanisms proposed to explain the phenomenon of nitrate tolerance, several studies have suggested a role of the cGMP-hydrolyzing PDEs, PDE1A1 (Kim et al., 2001) and PDE5 (Silver et al., 1991; Pagani et al., 1993; de Garavilla et al., 1996). Increased cGMP PDE activity would be expected to decrease cGMP levels and attenuate cGMP signaling. Given the preferential venodilator effects of GTN and reports suggesting the selective development of nitrate tolerance in the venous circulation (Stewart et al., 1986; Ghi et al., 1992; Bauer and Fung, 1996), we wished to examine the effects of PDE inhibition on the venodilator responses to GTN in nitrate-tolerant animals and compare these with effects on the arterial side of the circulation. We found a greater degree of tolerance in femoral veins from GTN-tolerant animals and that this was associated with a selective increase in cGMP PDE activity and a greater relative reversal of tolerance by the PDE5-selective inhibitor MBCQ. Furthermore, in in vivo studies, there was a selective reversal of tolerance to the CVP-lowering effect of GTN by zaprinast, a relatively selective inhibitor of PDE5.

The recent study of Kim et al. (2001) provided evidence for a role of the calcium/calmodulin-dependent PDE, PDE1A1, in nitrate tolerance. In aortae from GTN-tolerant rats, there was increased expression of PDE1A1, but not PDE5A1, at the mRNA and protein level and an increase in Ca/CAM-stimulated cGMP PDE activity. Furthermore, the PDE1-selective inhibitor, vinpocetine, lowered the EC_{50} for GTN-induced relaxation to a greater degree in aortae from GTN-tolerant animals compared with nontolerant animals. However, in contrast to the aorta, cGMP PDE activity was unaltered in the presence of Ca/CAM in either control or GTN-tolerant femoral artery or vein (Fig. 4), suggesting a minor role for PDE1 in cGMP catabolism in these blood vessels.

MBCQ is a potent and selective inhibitor of PDE5 with an IC_{50} value of 19 nM for PDE5 and >100 μM for other PDE isoforms (Takase et al., 1994). We found that this compound inhibited greater than 80% of the cGMP PDE activity in femoral artery and vein (Fig. 4), indicating that PDE5 is the dominant cGMP metabolizing PDE in these vessel types. Furthermore, the increment in cGMP PDE activity that was observed in homogenates of GTN-tolerant femoral vein was absent in samples incubated with MBCQ (Fig. 4B), suggesting that the increase in activity in tolerant vein was attributable to PDE5. The results of the isolated blood vessel relaxation studies are consistent with a role for increased PDE5 activity in the selective attenuation of GTN-induced vasodilatation in femoral vein. Although pretreatment of tissues with MBCQ resulted in an increase in sensitivity to GTN-induced relaxation in both femoral artery and vein from GTN-tolerant and nontolerant animals (Fig. 1), the greatest relative shift in potency occurred in tolerant femoral vein treated with MBCQ (Fig. 2).

The increase in cGMP PDE activity in tolerant vein and the effect of PDE inhibition by MBCQ to increase the sensitivity of tolerant veins to the relaxant effects of GTN suggests that, at least in venous tissues, a significant component of reduced cGMP signaling is due to increased cGMP catabolism rather than or in addition to reduced bioactivation of GTN or desensitization of sGC. One might therefore expect similar differences in responsiveness between femoral artery and vein with other compounds that act through cGMP signaling but that do not rely on enzymatic bioactivation. We assessed the effects of the spontaneous NO donor, DEA/NO, on the relaxation responses in GTN-tolerant and nontolerant tissues in the presence or absence of MBCQ (Fig. 3). Although MBCQ increased the sensitivity of both femoral artery and vein to the relaxant effects of DEA/NO, the EC_{50} values for relaxation were unchanged in blood vessels from GTN-tolerant animals, indicating minimal cross-tolerance between nitrates and spontaneous NO donors in these vessel types. This contrasts somewhat from data obtained in aorta from GTN-tolerant rats, in which partial cross-tolerance was observed for DEA/NO (there was approximately a 2-fold increase in the EC_{50} for DEA/NO in GTN-tolerant tissues versus a 6.5-fold increase for GTN; Ratz et al., 2000b).

Nitric oxide-induced vascular smooth muscle relaxation has both cGMP-dependent and -independent components. Cyclic GMP-independent mechanisms include activation of calcium-dependent potassium channels leading to membrane hyperpolarization (Bolotina et al., 1994) and stimulation of sarcoplasmic reticulum calcium ATPase activity resulting in increased calcium sequestration (Cohen et al., 1999). Thus, one possible explanation for the lack of cross-tolerance in femoral artery and vein could be redundancy in mechanisms of NO-dependent relaxation in these vessels such that cGMP-independent mechanisms of vasodilation are sufficiently robust to compensate for attenuated cGMP signaling. In other vessel types such as the aorta, there could be a relatively greater reliance on cGMP-dependent mechanisms for NO-induced relaxation, and this is reflected in the partial cross-tolerance observed.

In a previous study that examined the venodilator effects of GTN in intact, conscious animals, i.e. infusions of GTN had no effect on mean circulatory filling pressure (an index of total body venous tone) unless the animals had been pretreated with hexamethonium, indicating that the venodilator actions of GTN were masked by endogenous sympathetic tone (D'Oyley et al., 1989). Similar findings were obtained in the present study with respect to changes in CVP, and the use of hexamethonium allowed differences in the venodilator response to GTN in control and GTN-tolerant animals to be revealed (Fig. 6C) and also enhanced the differences in the MAP response in control and tolerant animals (Fig. 6D).

We chose to use zaprinast in the intact animal to allow comparison with previous in vivo studies that assessed the role of PDEs in nitrate tolerance (Silver et al., 1991; de Garavilla et al., 1996). Consistent with the data obtained in the in vitro experiments, administration of this cGMP PDE inhibitor at the dose chosen (1 mg/kg) selectively increased...
the sensitivity to the venodilator effects of GTN in GTN-tolerant animals (Fig. 7E), suggesting a selective up-regulation of PDE had occurred on the venous side of the circulation. Although zaprinast can inhibit PDE1 as well as PDE5, it is more selective for PDE5. This, together with the minimal effect of Ca/CAM on PDE activity in venous tissue (Fig. 4B) and the relatively greater abundance of PDE5 in venous tissue, suggests that the in vivo actions of zaprinast seen in our experiments were mediated by PDE5 inhibition. Our data differ from that of de Garavilla et al. (1996) in one respect. These authors induced tolerance by the s.c. administration of high-dose GTN (100 mg/kg i.t.) over a 2- to 3-day period and found that zaprinast doses between 0.1 and 10 mg/kg restored the MAP response to GTN in tolerant animals to control levels. We used a low-dose protocol for GTN tolerance induction (approximately 10-fold lower total dose) and found that 1 mg/kg zaprinast had no effect on MAP response to GTN in GTN-tolerant animals (Fig. 7F). The enhanced response to zaprinast could therefore be due to a greater degree of tolerance produced by the high-dose GTN protocol and could also be related to the use of anesthetized, spontaneously hypertensive rats in their study, which exhibited a much greater responsiveness to a given dose of GTN than did the conscious normotensive animals used here.

The immunoblot analysis of PDE5 indicated that there was no increase in PDE5 protein levels in femoral vein from tolerant animals (Fig. 5), suggesting mechanisms other than up-regulation of expression were responsible for the increase in cGMP PDE activity in response to chronic GTN exposure in vivo. Recent studies have demonstrated that PDE5 activation in platelets and smooth muscle cells is paralleled by phosphorylation of the protein on Ser92 (Mullershausen et al., 2001; Rybalkin et al., 2002) and that in smooth muscle cells, the protein is phosphorylated by activated PKG (Rybalkin et al., 2002). Thus, continuous stimulation of sGC during chronic GTN exposure might be expected to result in increased PDE5 phosphorylation mediated by PKG. Although activation of PDE5 by phosphorylation could explain the observed increase in PDE5 activity in the absence of changes in protein expression, whether this does occur and whether there is selective PDE5 phosphorylation in venous tissues remains to be resolved.

Nitrates tolerance is multifactorial in nature, and there is evidence for multiple mechanisms by which chronic exposure to nitrates results in altered function of both vascular smooth muscle and endothelial cells. Increased reactive oxygen and/or reactive nitrogen species (Gori and Parker, 2002), and the formation of protein nitrosative groups (Fung, 2004) have been proposed as initiating or triggering events that underlie many of the numerous changes that occur in the vasculature in response to chronic nitrate exposure. For example, increased superoxide may result in quenching of NO derived from GTN, increased endothelin-1 formation and consequent increased sensitivity to vasoconstrictors, uncoupling of endothelial NO synthesis, increased sympathetic activity, and alteration of GTN biotransformation enzymes. The thionitrate oxidation hypothesis provides an explanation for both the bioactivation of nitrates and inactivation of enzymes mediating bioactivation, the greater propensity for nitrates to induce tolerance relative to other nitrovasodilators and the lack of extensive cross-tolerance to other nitrovasodilators, and a mechanism for possible generation of reactive oxygen species. However, neither of these hypotheses provides an explanation for selective GTN tolerance in the venous circulation or for the selective increases in PDE5 activity observed in the current study.

In summary, our in vitro and in vivo data suggest that increased PDE5 activity contributes to the development of GTN tolerance, at least in the venous circulation. The exaggerated hypotensive response to clinically available PDE5 inhibitors in patients on nitrate therapy is well documented. Given that PDE5 inhibition restored the venodilator responses to GTN in GTN-tolerant animals and tissues, it is possible that judicious use of low dose PDE5 inhibitors could be useful for maintaining the clinically relevant venodilator responses to nitrates that are lost upon continuous exposure.

Acknowledgments

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Address correspondence to: Dr. B. M. Bennett, Department of Pharmacology and Toxicology, Faculty of Health Sciences, Queen’s University, Kingston, ON, Canada K7L 3N6. E-mail: brian.bennett@queensu.ca