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Evidence for a role of endothelin 1 and protein kinase C in nitroglycerin tolerance

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ABSTRACT **We sought to examine mechanisms responsible for increased vasoconstriction that occurs during development of nitroglycerin tolerance. Rabbits were treated for 3 days with nitroglycerin patches (0.4 mg/hr), and their aortic segments were studied in organ chambers. This treatment resulted in attenuated *in vitro* relaxations to nitroglycerin and increased contractile sensitivity to angiotensin II, serotonin, phenylephrine, KCl, and a direct activator of protein kinase C, the phorbol ester phorbol 12,13-dibutyrate. The protein kinase C antagonists calphostin C (100 nM) and staurosporine (10 nM) corrected the hypersensitivity to constrictors in tolerant vessels, yet had minimal effects on constrictions in control vessels. Paradoxically, constrictions caused by endothelin 1 were decreased in nitrate-tolerant vessels. Immunocytochemical analysis revealed intense endothelin 1-like and big endothelin 1-like immunoreactivity in the media of nitroglycerin-tolerant but not of control aortas. The enhanced vasoconstriction to angiotensin II, serotonin, KCl, and phenylephrine could be mimicked in normal vessels by addition of subthreshold concentrations of endothelin 1, and this effect was prevented by calphostin C. We propose that increased autocrine production of endothelin 1 in nitrate tolerance sensitizes vascular smooth muscle to a variety of vasoconstrictors through a protein kinase C-mediated mechanism.**

Chronic administration of organic nitrates invariably leads to the rapid development of nitrate tolerance (for review, see refs. 1 and 2). Traditional explanations for nitroglycerin tolerance have focused on mechanisms such as neurohumoral adaptations (3, 4), impaired nitroglycerin biotransformation to nitric oxide (5), or desensitization of the vascular smooth muscle target enzyme guanylyl cyclase (6). Recently, we have demonstrated that nitroglycerin tolerance is associated with an increase in vascular superoxide anion production, which likely inactivates endogenously released nitric oxide and nitric oxide released from exogenously applied nitrovasodilators (7). Together, these multiple mechanisms may account for decreased nitroglycerin-induced vasodilatation after its prolonged administration.

Much less attention has been devoted to the possible role of enhanced vasoconstriction in nitrate tolerance. Previous studies have shown that long-term treatment with high doses of nitroglycerin is associated with an increase in sensitivity to α-adrenergic stimuli (8, 9). Further, abrupt cessation of nitrate therapy often leads to rebound vasoconstriction (10), which may be due to an increased sensitivity of the nitrate-tolerant vasculature to endogenous vasoconstrictors. The mechanisms underlying these phenomena remain obscure.

In the present study we sought to determine whether chronic nitroglycerin treatment, in clinically relevant concentrations, increases sensitivity to constrictors and to examine mechanisms potentially responsible for this phenomenon.

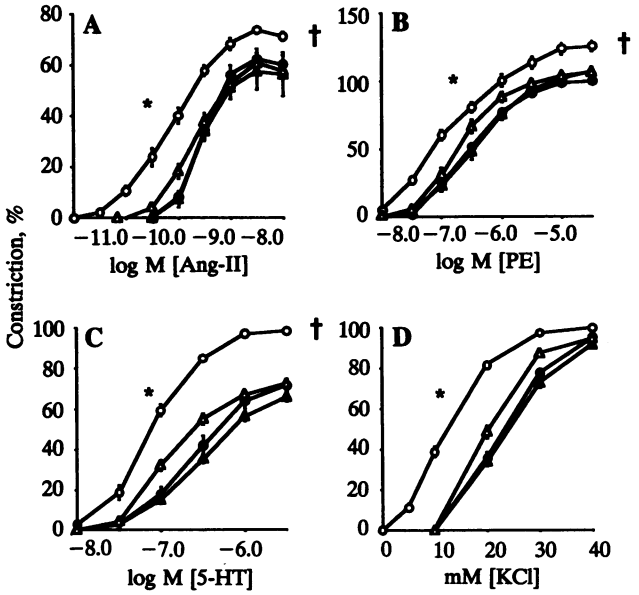

FIG. 1. Effects of 3 days of nitroglycerin treatment on sensitivity to Ang-II (*A*), phenylephrine (PE) (*B*), 5-HT (*C*), and KCl (*D*). Nitrate tolerance was associated with an increase in sensitivity to all constrictors, which was largely corrected by calphostin C (100 nM). Each value represents the mean ± SEM of 8–13 experiments, except for calphostin C, which represents 5–7 experiments. ●, Control; ▲, control + 10^{-7} M calphostin C; ○, nitrate tolerant; △, nitrate tolerant + 10^{-7} M calphostin C. *, EC_{50} ($P < 0.05$ vs. control). †, Maximum constriction ($P < 0.05$ vs. tolerant).

METHODS AND MATERIALS

Animal Model. New Zealand White rabbits of either sex (weight = 3–6 kg) were studied. A region either on the dorsal aspect of the thorax or between the scapulae was shaved, and a nitroglycerin patch was applied to the skin. The nitroglycerin patch was changed each morning for 2 days. On the morning of the third day, an i.v. injection of 100 units of heparin was administered, followed by a lethal dose of pentobarbital. The chest was then rapidly opened, and the descending thoracic aorta was removed. Rabbits of a similar size and sex served as controls.

Vessel Preparation and Organ Chamber Experiments. Organ chamber studies were done as described (7). Relaxations to nitroglycerin were evoked after the vessels were submaxi-

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Abbreviations: PBt_2, phorbol 12,13-dibutyrate; Ang-II, angiotensin II; 5-HT, serotonin; PKC, protein kinase C.

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Table 1. Effects of PKC inhibition on EC_{50} of various vasoconstrictors in control and tolerant rabbit aorta

	Ang-II, −log M	PE, −log M	5-HT, −log M	KCl, mM
Control aorta	9.06 ± 0.06	6.58 ± 0.05	6.64 ± 0.08	23 ± 1
Calphostin C (100 nM)	9.20 ± 0.08	6.51 ± 0.09	6.57 ± 0.07	24 ± 1
Staurosporine (10 nM)	8.73 ± 0.08†	6.64 ± 0.06	6.51 ± 0.01	23 ± 1
Tolerant aorta	9.76 ± 0.11*	6.97 ± 0.07*	7.16 ± 0.05*	12 ± 1*
Calphostin C (100 nM)	9.21 ± 0.08†	6.69 ± 0.06†	6.84 ± 0.06†	20 ± 1†
Staurosporine (10 nM)	8.70 ± 0.07†	6.67 ± 0.05†	6.82 ± 0.07†	19 ± 1†

The sensitivities of Ang-II, phenylephrine (PE), 5-HT, and KCl are expressed as EC_{50} (concentration that produces 50% of the maximal contraction). Each value represents the mean ± SEM of 8–13 experiments, except for PKC-inhibitor treatment, which is equal to 4–7 experiments.
*P = 0.05 tolerant vs. control.
†P = 0.05 vs. without calphostin C or staurosporine.

mally constricted with phenylephrine. Contractions to a range of doses of phenylephrine, angiotensin II (Ang-II), serotonin (5-HT), and the phorbol ester phorbol 12,13-dibutyrate (PBt_2) were obtained and quantified as a percentage of the contractions to a maximal depolarizing concentration (80 mM) of KCl. In some studies, to inhibit protein kinase C (PKC), either calphostin C or staurosporine was applied 60 min before the cumulative concentration–response curves. All experiments were done under ordinary fluorescent light because of the photoactivatable properties of calphostin C (11).

Immunocytochemical Analysis. Two polyclonal antisera against human endothelin 1 were used as described recently (12), one against the C terminus of endothelin 1 and the other against the C-terminal fragment of big endothelin 1 [big endothelin-(22–38)]. A commercial antiserum against human endothelin 1 (Peninsula Laboratories) was also used. In addition, antiserum to von Willebrand factor (factor VIII-related antigen) (Dako) was used as an endothelial cell marker. The avidin–biotin–peroxidase complex method was used as previously described (12). Negative controls were prepared with the specific antiserum absorbed with the cross-reactive endothelins or with nonimmune serum instead of primary antiserum, or by omitting steps in the avidin–biotin–peroxidase procedure. For each antiserum, three sections were stained.

Materials. Phenylephrine, PBt_2, calphostin C, staurosporine, Ang-II, endothelin 1, dimethyl sulfoxide, and KCl were all purchased from Sigma. Nitroglycerin was supplied by DuPont. All agents were dissolved in distilled water except PBt_2 and calphostin C, which were dissolved in dimethyl sulfoxide.

Statistical Analysis. Results are expressed as means ± SEMs. The ED_{50} value for each experiment was obtained by logarithmic transformation. Vascular responses were compared by using ANOVA with ED_{50} and maximal dilations or constrictions as dependent variables. A Student–Newman–Keuls post hoc test was used when significance was indicated. Significance was assumed present when $P < 0.05$.

RESULTS

Effect of Chronic Nitroglycerin Therapy on Relaxations Caused by Nitroglycerin. Treatment with nitroglycerin for 3 days resulted in a marked inhibition of nitroglycerin-mediated vasodilation. Maximal relaxations were 92 ± 5% in control vessels and 56 ± 4% in tolerant vessels, confirming the presence of nitrate tolerance.

Effect of Nitrate Tolerance on Responses to Ang-II, Phenylephrine, 5-HT, and KCl. In preliminary experiments, constrictions to phenylephrine and Ang-II were not only enhanced but also unusually sustained. Even with repeated washings, the time to complete relaxation after constriction caused by either Ang-II or phenylephrine averaged 38 ± 3 min and 48 ± 4 min in nitrate-tolerant vessels vs. 16 ± 4 and 22 ± 3 min in control vessels (n = 5 each). Sustained vasoconstrictions occur in response to activation of PKC (for review, see ref. 13). Pretreatment of nitroglycerin-tolerant aortas for 1 hr with either calphostin C (100 nM) or staurosporine (10 nM) normalized contractions caused by either Ang-II, phenylephrine, 5-HT, or KCl. In contrast, these PKC inhibitors had only modest or no effect on constrictions to these vasoconstrictor agents in normal vessels (Fig. 1 and Tables 1 and 2). In addition, constrictions induced by a direct activator of PKC, the phorbol ester PBt_2, were markedly enhanced in tolerant aortic segments (Fig. 2).

Effect of Nitrate Tolerance on Constrictions Caused by Endothelin 1. In contrast to the findings with other vasoconstrictors, responses to endothelin 1 were attenuated in aortic segments from nitroglycerin-treated animals as compared with those from control animals (Fig. 3).

Endothelin 1 and Big Endothelin 1 Localization in Normal and Tolerant Vessels. Immunocytochemical analysis of control rabbit aortas revealed no endothelin 1-like immunoreactivity staining of the media (Fig. 4). In contrast, the aorta of nitro-

Table 2. Effects of PKC inhibition on maximal increases in tension to various vasoconstrictors of control and nitrate-tolerant rabbit aorta

	Potency, % maximal response to KCl			
	Ang-II	PE	5-HT	KCl potency, g
Control aorta	63 ± 2	100 ± 3	72 ± 3	7.8 ± 0.6
Calphostin C (100 nM)	53 ± 6	105 ± 3	66 ± 2	7.4 ± 0.3
Staurosporine (10 nM)	56 ± 4	99 ± 6	56 ± 5†	7.5 ± 0.4
Tolerant aorta	74 ± 2*	127 ± 4*	99 ± 2*	8.5 ± 0.5
Calphostin C (100 nM)	62 ± 2†	107 ± 2†	72 ± 2†	8.3 ± 0.3
Staurosporine (10 nM)	56 ± 4†	107 ± 5†	79 ± 3†	7.6 ± 0.6

The constrictor potencies of Ang-II, phenylephrine (PE), and 5-HT are expressed as percentage of the maximal response to 80 mM KCl; the potency of KCl itself is expressed in grams. Each value represents mean ± SEM of 8–13 experiments, except for PKC-inhibition experiments, which are n = 4–7.
*$P < 0.05$ tolerant vs. control.
†$P < 0.05$ vs. without calphostin C or staurosporine.

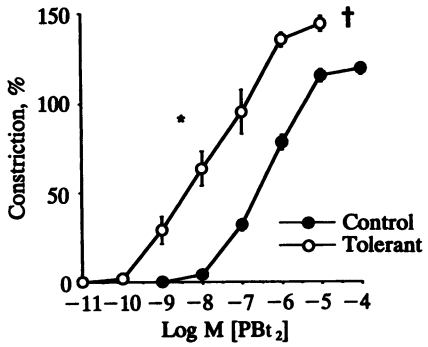

FIG. 2. Effect of 3 days of nitroglycerin treatment on constrictions induced by the phorbol ester PBt_2 (n = 8 tolerant and 11 control). *, EC_{50} ($P < 0.05$ vs. control). †, Maximum constriction ($P < 0.05$ vs. control).

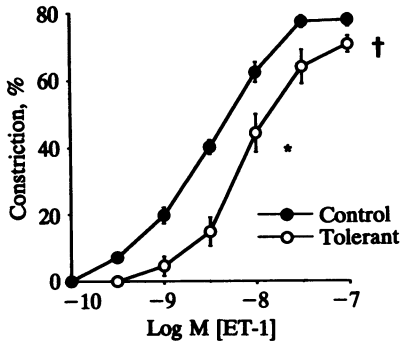

FIG. 3. Effect of 3 days of nitroglycerin treatment on constrictions induced by endothelin 1 (ET-1) (n = 8 for control and 11 for nitroglycerin tolerant animals). *, EC_{50} ($P < 0.05$ vs. control). †, Maximum constriction ($P < 0.05$ vs. control).

glycerin-treated animals showed intense endothelin 1 staining. Similar findings were observed for big endothelin 1-like immunoreactivity (Fig. 4).

Effect of Endothelin 1 on Vasoconstrictor Responses to Other Stimuli. Preincubation of control aortic segments with threshold concentrations of endothelin 1 (3×10^{-10} M) for 10 min before and during administration of KCl, phenylephrine, Ang-II, and 5-HT markedly enhanced constriction to each of these agents. This increased sensitivity to these constrictors caused by endothelin 1 was prevented by calphostin C addition (Fig. 5).

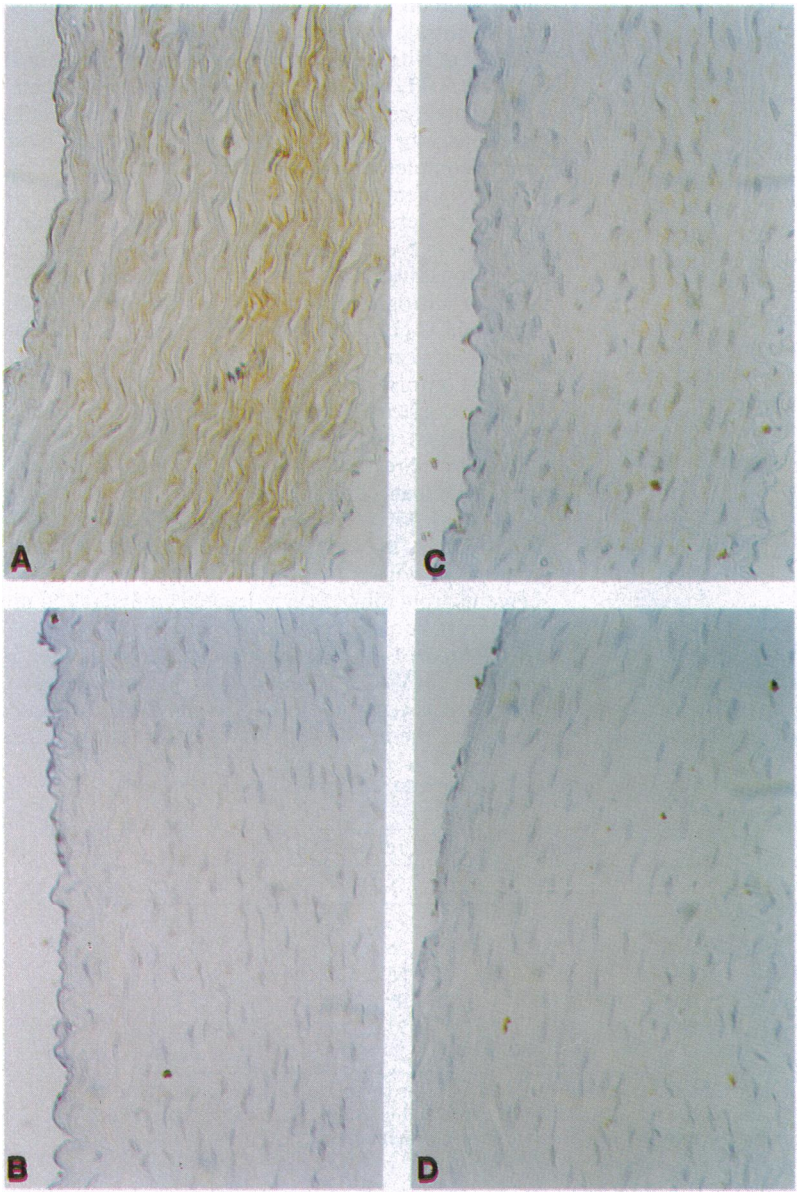

FIG. 4. Endothelin 1 (*A* and *B*) and big endothelin 1 (*C* and *D*) immunoreactivity in rabbit aortic segments. In nitroglycerin-tolerant (*A* and *C*) segments, both endothelin 1 and big endothelin 1 immunoreactivity (brown stain) were present in the media. Normal (*B* and *D*) rabbit aortas did not exhibit positive staining for either endothelin 1 or big endothelin 1. Figures are representative of six control and six nitroglycerin-treated rabbits examined similarly. (×900.)

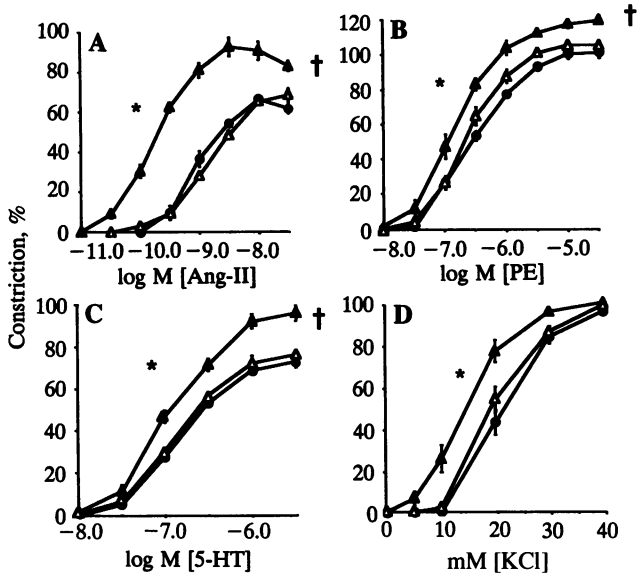

FIG. 5. Effects of preincubation of normal aortic segments with endothelin 1 (10 min) on contractions caused by Ang-II (*A*), phenylephrine (*B*), 5-HT (*C*) and KCl (*D*), in the presence and absence of calphostin C. (n = 4 or 5 for each series of dose–response curves). ●, Control; ▲, control + 3×10^{-10} M endothelin 1; △, control + 3×10^{-10} M endothelin 1 and 10^{-7} M calphostin C. *, EC_{50} ($P < 0.05$ vs. control). †, Maximum constriction ($P < 0.05$ vs. tolerant).

DISCUSSION

In the present studies, we have defined a mechanism by which vascular responsiveness may be altered during prolonged nitrate therapy. Nitrate tolerance was associated not only with decreased vasodilatation in response to nitroglycerin but also with increased vasoconstriction to a variety of neurohumoral agents. The enhanced nature of these constrictions was shared by a direct activator of PKC and was prevented by PKC inhibitors. Paradoxically, constrictions to endothelin 1 were decreased in nitrate-tolerant vessels compared with controls. Using immunocytochemical techniques, we identified intense staining for endothelin 1 and big endothelin in the media of the nitrate-tolerant vessels. In separate studies, the acute addition of low concentrations of endothelin 1 to normal vessels increased contractions to several neurohumoral agonists in a manner mimicking that observed in nitrate-tolerant vessels. We propose that in nitrate tolerance, autocrine-produced endothelin 1 sensitizes the vasculature to vasoconstrictors by a PKC-dependent mechanism.

It has previously been suggested that nitroglycerin tolerance is associated with a specific enhancement of α-adrenergic receptor-mediated contractions to epinephrine and norepinephrine (8, 9). In the present study, chronic nitroglycerin treatment markedly increased responses to almost all vasoconstrictors studied. Thus, enhanced contractile sensitivity in nitrate tolerance is not specific to any one agonist but likely involves an intracellular signaling process shared by the various receptors activated by these agonists.

An interesting finding in the present study was that constrictions of nitrate-tolerant vessels in response to Ang-II or phenylephrine were sustained even after washing the various agents from the organ chambers. Vascular contractions mediated by activation of PKC are known to be sustained (13). This result prompted us to consider an enhanced involvement of PKC in nitrate-tolerant vessels. Two structurally different PKC inhibitors, calphostin C [an inhibitor for the regulatory domain (14)] and staurosporine [an inhibitor of the catalytic domain (15)] corrected hypersensitivity of nitrate-tolerant vessels to all constrictors but had minimal effects in control vessels. Additionally, the phorbol ester PBt_2 produced markedly greater constriction in nitrate-tolerant vs. control vessels, further supporting a role for PKC in nitrate tolerance.

Like the other vasoconstrictors studied, endothelin 1 activates PKC (16). Surprisingly, contractions caused by endothelin 1 were attenuated in nitrate-tolerant aortic segments. One explanation for this finding could be related to binding of existing receptors by locally produced endothelin. Indeed, vessels from rabbits treated for 3 days with nitroglycerin exhibited striking endothelin 1 and big endothelin 1 staining within the media, a finding not observed in control aortas. Increases in local endothelin 1 might either downregulate or occupy endothelin 1 receptors, making them unavailable for activation by exogenous endothelin (17).

The mechanism whereby nitrate tolerance could increase local endothelin 1 production remains unclear. In cultured vascular smooth muscle, low concentrations of Ang-II induce expression of pre-pro-endothelin mRNA via stimulation of the Ang type-1 receptor in a PKC- dependent mechanism (18, 19). The renin/angiotensin system is activated during nitrate therapy (20) and may, therefore, contribute to stimulation of vascular smooth muscle endothelin 1 expression.

What is the link between increased local endothelin concentrations and increase in sensitivity to constrictors? In the present studies we found that threshold endothelin 1 concentrations (3×10^{-10} M) markedly enhanced constrictions caused by Ang-II, 5-HT, KCl, and phenylephrine. These findings agree with previous studies in human coronary arteries showing that threshold concentrations of endothelin may markedly enhance vasoconstrictions caused by 5-HT and norepinephrine (21). Similarly, Henrion and Laher (22) have shown that endothelin 1 potentiates norepinephrine-induced contractions in rabbit aorta and that this effect can be prevented by PKC inhibition. On the basis of our current findings and these prior studies, it is likely that endothelin 1, either acutely added to vessels or expressed in the vascular media during nitroglycerin treatment, enhances constrictions to many vasoconstrictor agents via activation of PKC.

A role of PKC in augmenting vasoconstriction in nitrate-tolerant vessels is compatible with accumulating information regarding its role in modulating vascular tone. Myogenic tone is decreased by PKC inhibition (23) and increased by PKC activation (24). Phorbol esters convert the transient constrictions caused by Ang-II in rat aorta to sustained responses (25). The mechanisms responsible for these phenomena remain uncertain. Activation of one or more of the vascular smooth muscle isoforms of PKC results in phosphorylation of several contractile proteins—including myosin light and heavy chains (26, 27), myosin light chain kinase (28), and the intermediate filaments desmin, synemin, and caldesmon (29). Activation of voltage-gated calcium channels has also been implicated (30). Redistribution of the thin-filament calponin among cytoskeletal structures after α-adrenergic stimulation also depends on PKC (31). An important consequence of these events is that the contractile response to any given level of intracellular calcium is increased (24, 32). In several cell types, stimulation of PKC by phorbol esters leads to activation of phospholipase C and accumulation of diacylglycerol, which may further activate PKC in a positive feedback fashion (33).

We conclude that chronic nitroglycerin treatment increases endothelin 1 levels within the vascular smooth muscle. This increase in vascular endothelin 1 serves as a "priming" stimulus for PKC which, in turn, mediates hypersensitivity to a variety of vasoconstrictor stimuli. At least two other conditions of altered vascular reactivity, advanced atherosclerosis, and pulmonary hypertension (12, 34) are also associated with increased vascular levels of endothelin 1. Thus, nitrate tolerance may share with these diseases a common mechanism underlying increased sensitivity to vasoconstrictor stimuli.

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