Upregulation of Phosphodiesterase 1A1 Expression Is Associated With the Development of Nitrate Tolerance

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Background—The efficacy of nitroglycerin (NTG) as a vasodilator is limited by tolerance, which develops shortly after treatment begins. In vascular smooth muscle cells (VSMCs), NTG is denitrated to form nitric oxide (NO), which activates guanylyl cyclase and generates cGMP. cGMP plays a key role in nitrate-induced vasodilation by reducing intracellular Ca²⁺ concentration. Therefore, one possible mechanism for development of nitrate tolerance would be increased activity of the cGMP phosphodiesterase (PDE), which decreases cGMP levels.

Methods and Results—To test this hypothesis, rats were made tolerant by continuous infusion of NTG for 3 days (10 μg · kg⁻¹ · min⁻¹ SC) with an osmotic pump. Analysis of PDE activities showed an increased function of Ca²⁺/calmodulin (CaM)–stimulated PDE (PDE1A1), which preferentially hydrolyzes cGMP after NTG treatment. Western blot analysis for the Ca²⁺/CaM-stimulated PDE revealed that PDE1A1 was increased 2.3-fold in NTG-tolerant rat aortas. Increased PDE1A1 was due to mRNA upregulation as measured by relative quantitative reverse transcription–polymerase chain reaction. The PDE1-specific inhibitor vinpocetine partially restored the sensitivity of the tolerant vasculature to subsequent NTG exposure. In cultured rat aortic VSMCs, angiotensin II (Ang II) increased PDE1A1 activity, and vinpocetine blocked the effect of Ang II on decrease in cGMP accumulation.

Conclusions—Induction of PDE1A1 in nitrate-tolerant vessels may be one mechanism by which NO/cGMP-mediated vasodilation is desensitized and Ca²⁺-mediated vasoconstriction is supersensitized. Inhibiting PDE1A1 expression and/or activity could be a novel therapeutic approach to limit nitrate tolerance. (Circulation. 2001;104:2338-2343.)

Key Words: nitrates ▪ phosphodiesterase ▪ vasculature

Nitroglycerin (NTG) remains one of the foremost drugs in the treatment of angina pectoris. When given in the short term, NTG has potent vasodilator capacities on arteries, veins, and coronary collateral vessels.¹ NTG induces vasorelaxation by releasing NO. NO activates soluble guanylyl cyclase and subsequently increases cGMP. cGMP in turn activates a CGMP-dependent protein kinase that has been shown to mediate vasorelaxation via phosphorylation of proteins that regulate intracellular Ca²⁺ levels.² The efficacy of chronic NTG administration, however, is limited by the rapid development of tolerance,³ involving decreased vascular sensitivity and diminished cGMP elevations in vascular smooth muscle cells (VSMCs) in response to continued nitrate treatment. Nitrate tolerance is also associated with cross-tolerance to other endothelium-dependent and -independent vasodilators.² Several mechanisms have been proposed to account for this phenomenon, such as neurohumoral counterregulation⁴ or mechanisms intrinsic to the vascular tissue itself, such as desensitizing NO/cGMP signaling and responses.² Chronic NTG treatment has also been shown to be associated with an increase in sensitivity to vasoconstrictors such as catecholamines, angiotensin (Ang) II, KCl, and serotonin,⁵ all of which may compromise the vasodilating capacity of NTG, thereby contributing to tolerance. Another aspect of organic nitrate therapy is the development of rebound ischemia due to coronary rebound constriction after abrupt cessation of long-term NTG therapy.⁶

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cGMP appears to be a key player in NTG-mediated vasorelaxation, and the cGMP level is highly regulated by phosphodiesterase (PDE). At least 4 different major PDE activities have been identified in VSMCs (Table). Different PDEs play distinct roles in controlling vascular tone.⁷ In this study, we found that the activity and expression of Ca²⁺/calmodulin (CaM)–stimulated PDE (PDE1A1), which preferentially hydrolyzes cGMP, were induced by NTG treatment. Vinpocetine, a selective inhibitor of the PDE1 family, was
able to partially restore the sensitivity of tolerant vessels to subsequent NTG exposure. These findings indicate that increased PDE1A1 activity can decrease cGMP levels, which may contribute at least in part to the attenuation of the NTG-mediated vasodilation as well as the supersensitivity to vasoconstrictors.

Methods

Development of Nitrate Tolerance In Vivo
Nitrate tolerance was induced as described previously. Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, Mass; 250 to 300 g) were anesthetized with ketamine 40 mg/kg, xylazine 0.5 mg/kg, and acepromazine 5 mg/kg IP. An osmotic minipump (model 2 ML1, Alza Corp) filled with either NTG (Zeneca Inc) or vehicle (propylene glycol) was implanted subcutaneously at the dorsum of the neck. NTG was infused at a rate of 10 μg·kg⁻¹·min⁻¹ for 3 days.

To assess tolerance, mean arterial pressure (MAP) was continuously monitored by a catheter in the right common carotid artery on a polygraph recorder (Grass Instruments). Bolus doses of NTG or hydralazine were administered through the right jugular vein.

Anion-Exchange Chromatography
Three thoracic aortas were pooled and homogenized. The supernatant of the tissue extract was fractionated as described previously. Fractions containing PDEs were assayed for PDE activity or subjected to Western blot analysis.

In Vitro PDE Enzyme Assay
PDE assays were carried out according to established procedures.

Western Blot Analysis
Western blots for PDE1A1 and PDE5A1 with isoform-specific antibodies were carried out as described previously.

Relative Quantitative RT-PCR and Northern Blot Analysis
Tissue RNA was extracted with a Total RNA Isolation Kit (Ambion). First-strand cDNA was synthesized with the SuperScript Preamplification System (Gibco-BRL). Relative quantitative reverse transcription–polymerase chain reaction (RT-PCR) was performed with 18s rRNA as an internal control by use of Ambion’s competimer technology. Isoform-specific primers were used to generate the 281-bp PCR product for PDE1A1 or 335-bp PCR product for PDE5A1. PCR products were separated on a 2% agarose gel, stained with ethidium bromide, and visualized under UV light. The relative intensity of PDE1A1 or PDE5A1 PCR products was determined by densitometry. Northern blot analysis was performed with the Clontech hybridization system according to the manufacturer’s protocol. The PCR product specific to PDE1A1 or PDE5A1, mentioned above, was used as a probe.

Contraction Study
Ex vivo evaluation of nitrate tolerance and reversal was performed as described previously. Aortic rings were stretched with a preload of 2 g, equilibrated, and then preconstricted with phenylephrine. Preconstricted aortic rings were pretreated with vehicle or 100 μmol/L vinpocetine for 10 minutes, followed by exposure to ascending concentrations of NTG.

cGMP Assays
Aortic extracts were prepared by homogenization in cold 5% trichloroacetic acid. VSMC extracts were prepared by lysis of cells in cold 100% ethanol. A cGMP [3H]radioimmunoassay kit (NEN) was used for measurement of cGMP levels as described in the manufacturer’s protocol. Protein levels were determined by the method of Bradford (Bio-Rad).

VSMC Culture
Rat aortic VSMCs were isolated and maintained as described previously. VSMCs (passages 6 to 9) were growth-arrested for 48 hours before the indicated drug treatment.

Measurement of Ca²⁺/CaM-Stimulated PDE Activity
The technique involves extraction and assay of enzyme activity at a low temperature and in the presence of trifluoperazine to prevent the dissociation and reassociation of the ternary Ca²⁺-CaM-PDE complex during cell disruption. The percent maximal CaM-stimulated PDE activity was computed as (activity without CaM or EGTA–activity with EGTA)/(activity with CaM–activity with EGTA)×100.

Statistical Analysis
One- or 2-way ANOVA was used to compare differences between treatment means (expressed as mean±SEM). After ANOVA, comparison of 2 populations was made by Student’s unpaired t test. A value of P<0.05 was considered significant.

Results

Induction of In Vivo Nitrate Tolerance
To assess the development of nitrate tolerance in vivo, the hypotensive and vasorelaxant effects of NTG were examined. There was no significant difference in baseline MAP between the vehicle- and NTG-treated groups (104.3±3.9 versus 100.8±4.1 mm Hg). In the vehicle-treated group, acute NTG challenges caused a dose-dependent drop in MAP (Figure 1), but 3-day NTG treatment significantly decreased the NTG-induced drop in MAP (Figure 1), indicating that changes in MAP were significantly blunted in the NTG-treated groups. The hydralazine (1 mg/kg bolus)–induced drop in MAP was similar in both groups (Figure 1), indicating that NO-independent vasodilation was not different between the 2 groups.
Increased Ca\(^{2+}\)/CaM-Stimulated PDE Activity in Nitrate-Tolerant Rat Aortas

To examine the change of PDE activity in nitrate-tolerant vessels, we performed PDE assays. cGMP hydrolytic activities in tolerant aortas were significantly higher than in control aortas (Figure 2A), whereas cAMP hydrolytic activities were similar (Figure 2B). In the presence of Ca\(^{2+}\)/CaM, cGMP hydrolytic activities were increased much more in tolerant aortas than in control aortas (Figure 2A). This observation suggests that the activity of a Ca\(^{2+}\)/CaM-stimulated PDE, which preferentially hydrolyzes cGMP, is induced by NTG treatment.

To confirm these results and further separate different PDE activities, we resolved the PDE activities of control and tolerant aortas by high-performance liquid chromatography. In fractions 18 to 30, which contain enzymes of both the PDE1 and PDE5 families,\(^9\) cGMP hydrolytic activities were increased more in tolerant aortas than in control aortas (Figure 2A). This observation suggests that the activity of a Ca\(^{2+}\)/CaM-stimulated PDE, which preferentially hydrolyzes cGMP, is induced by NTG treatment.

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Protein Level of PDE1A1, but Not PDE5A1, Is Increased in Nitrate-Tolerant Aortas

To determine whether the induction of PDE activity in tolerant vessels was due to increases in the enzyme level, Western blot analyses with PDE1A- and PDE5A-specific antibodies were performed (Figure 3). The PDE1A1 protein level was increased 2.3-fold in tolerant vessels (Figure 3A), whereas the PDE5A1 protein level was not significantly changed (Figure 3B). These results indicate that PDE1A1, a Ca\(^{2+}\)/CaM-stimulated PDE with much higher affinity for cGMP than for cAMP,\(^14\) is selectively upregulated in nitrate-tolerant vessels.

mRNA Level of PDE1A1, but Not PDE5A1, Is Increased in Nitrate-Tolerant Aortas

To determine whether the increase in protein level of PDE1A1 was due to an increase in its mRNA level, relative quantitative RT-PCRs using PDE1A1- and PDE5A1-specific primers were performed (Figure 4). mRNA levels of PDE1A1 in tolerant aortas were increased by 2.4-fold compared with control aortas (Figure 4A). In contrast, the PDE5A1 mRNA...
level was not significantly altered (Figure 4B). We obtained similar results by Northern blot analysis (Figure 4C).

**Effects of PDE1 Family–Selective Inhibitor Vinpocetine on Reversal of Nitrate Tolerance Ex Vivo**

NTG-treated aortic rings exhibited a significantly decreased vasorelaxation response to subsequent NTG compared with control. Preincubation of tolerant rings with vinpocetine, however, restored the vasorelaxant effect of NTG. Vinpocetine also potentiated the vasorelaxant effect of NTG in control rings (Figure 5A).

We also tested the effects of vinpocetine on nitrate tolerance–associated attenuation of cGMP elevations in response to subsequent NTG exposure as shown in Figure 5B. The basal cGMP concentrations were not significantly different between tolerant and control aortic rings. After exposure to NTG, an NTG-induced increase in cGMP in tolerant rings was clearly decreased compared with control, and vinpocetine enhanced the NTG-induced increase in cGMP in tolerant rings, suggesting that PDE1A1 inhibitor is able to partially reverse the nitrate tolerance of cGMP response.

**Stimulation of PDE1A1 Activity by Ang II and the Role of PDE1A1 in Ang II–Mediated Inhibition of Atrial Natriuretic Peptide–Evoked cGMP Accumulation in VSMCs**

To study the mechanism by which induction of PDE1A1 might cause nitrate tolerance and nitrate tolerance–induced supersensitivity to vasoconstrictors, we measured the effects of Ang II on PDE1A1 activity, which represent the extent of activation in vivo. As shown in Figure 6A, PDE1A1 activity in control VSMCs is 47.8% of the maximum. Ang II increased PDE1A1 activity to 95.6% of the maximum. This observation is consistent with the time course of force production and changes in intracellular Ca2+/H11001 concentration in response to Ang II treatment 15 and suggests that Ang II is able to stimulate the PDE1A1 activity, probably via an Ang II–mediated increase in Ca2+/H11001 concentration.

To further determine the functional effects of PDE1A1 activity stimulated by Ang II, we measured the effect of Ang II on cGMP accumulation in response to atrial natriuretic peptide (ANP). The addition of ANP to VSMCs rapidly increased intracellular cGMP. Simultaneous addition of Ang II markedly decreased the ANP-induced cGMP accumulation. Vinpocetine significantly blocked the inhibitory effect of Ang II (Figure 6B). These results suggest that PDE1A1 in
Nitrate tolerance

Figure 7. Schematic showing role of PDE1A1 in nitrate tolerance. Endothelium-dependent and -independent nitrovasodilators such as acetylcholine (Ach), NTG, and ANP activate soluble guanylyl cyclase (GC), stimulating cGMP formation. cGMP leads to vasodilation by decreasing intracellular Ca\(^{2+}\). Vasoconstrictors such as Ang II, norepinephrine (NE), and endothelin (ET)-1 cause increase in intracellular Ca\(^{2+}\), leading to vasoconstriction. cGMP is a key player in regulating vascular tone by controlling Ca\(^{2+}\) levels, which regulates vascular contractility. An increase in PDE1A1 activity by chronic NTG treatment leads to reduced cGMP accumulation in response to further stimulation with vasoconstrictors, thereby causing a decreased sensitivity of vasculature to NTG. Induction of PDE1A1 also increases magnitude of vasoconstrictor-mediated attenuation of cGMP accumulation, which may contribute to phenomenon of supersensitivity to vasoconstrictors in NTG-treated vessels.

VSMCs plays a major role in mediating inhibition of ANP-evoked cGMP accumulation by Ang II.

Role of PDE1A1 in Nitrate Tolerance

PDE1A1 was previously shown to be important for the regulation of vascular cGMP levels and reactivity.\(^7,16\) The findings of the present study concur with this concept. We found that NTG treatment significantly increases PDE1A1 activity and expression. An increase in PDE1A1 activity will reduce cGMP accumulation, thereby causing the decreased sensitivity of the vasculature to subsequent NTG exposure; and third, that PDE1A1 plays an important role in regulation of intracellular cGMP levels in response to vasoconstrictors. The upregulation of PDE1A1 may therefore provide a new mechanism to explain, at least in part, the decreased sensitivity of the vasculature to NTG and the phenomenon of enhanced vasoconstriction observed after chronic NTG treatment (Figure 7).

Mechanisms Underlying Nitrate Tolerance

Nitrate tolerance seems to be multifactorial. Several mechanisms have been proposed to explain the phenomenon of nitrate tolerance. As a vasodilator, NTG lowers blood pressure, which in turn activates neurohumoral counterregulatory mechanisms, such as an activation of the renin-angiotensin system, increases in vasopressin levels, intravascular volume expansion, and increases in catecholamine release. These circulating vasoconstrictor forces may limit NTG-mediated vasodilation (pseudotolerance). Induction of PDE1A1 would enhance the vasoconstrictor forces.
Studies with isolated vessels from NTG-treated animals also show decreased sensitivity to NTG in the absence of the neurohumoral environment, pointing to intrinsic abnormalities of the tolerant vasculature itself. In particular, multiple steps in NO/cGMP signaling have been found to be affected. These include impaired nitrate biotransformation2; overproduction of reactive oxygen species, which reduces NO bioavailability;17 decrease and increase of cGMP metabolic enzymes, such as guanylyl cyclase and PDE, respectively;19,23; and attenuation of the downstream cGMP-dependent protein kinase activity.24 Our observation that inhibition of PDE1A1 partially restores the cGMP elevation and vasorelaxation to subsequent NTG exposure in tolerant vessels is consistent with the proposal that nitrate tolerance is contributed to by multiple factors.

Effects of PDE Inhibition on Tolerance

PDE1 isoforms are structurally closer to PDE5 isoforms than any other cAMP-hydrolyzing PDE isoforms in VSMCs. Zaprinast, which inhibits both PDE1 and PDE5 isoforms with higher sensitivity to PDE5, has been shown to be able to reverse NTG tolerance in vitro and in vivo.11,12 The effect of zaprinast on the reversal of nitrate tolerance may be due to the inhibition of both PDE1A1 and PDE5A1. Inhibition of PDE5A1 activity is able to diminish nitrate tolerance because of augmentation of the response to organic nitrates, even though PDE5A1 expression is not altered in the setting of nitrate tolerance.

Vinpocetine is the most selective inhibitor identified to date for PDE1 isoforms. Using recombinant PDE1A1 and PDE5A1 expressed in COS-7 cells, we found that vinpocetine at 100 μmol/L inhibited PDE1A1 activity up to 90% but PDE5A1 activity <5% (data not shown). This is consistent with the previous observation that the IC50 for vinpocetine to inhibit the partially purified PDE5A1 was >1 mmol/L.16 Thus, vinpocetine at a concentration of 100 μmol/L appears to be specific to PDE1A1 in VSMCs. In this study, we have demonstrated the ability of vinpocetine to limit nitrate tolerance in both restoration of vasorelaxation and cGMP response to NTG exposure in tolerant vessels, which suggests that PDE1A1 plays an important role in regulation of vascular reactivity, and also that upregulation of PDE1A1 due to chronic NTG treatment at least partially contributes to nitrate tolerance.

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References

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