Effects of In Vivo Nitroglycerin Treatment on Activity and Expression of the Guanylyl Cyclase and cGMP-Dependent Protein Kinase and Their Downstream Target Vasodilator-Stimulated Phosphoprotein in Aorta

Alexander Mülsch, PhD; Matthias Oelze, BS; Stefan Klöss, PhD; Hanke Mollnau, MD; Andrea Töpfer, PhD; Albert Smolenski, MD; Ulrich Walter, MD; Johannes-Peter Stasch, MD; Ascan Warnholtz, MD; Ulrich Hink, MD; Thomas Meinertz, MD; Thomas Münzel, MD

Background—Chronic in vivo treatment with nitroglycerin (NTG) induces tolerance to nitrates and cross-tolerance to nitrovasodilators and endothelium-derived nitric oxide (NO). We previously identified increased vascular superoxide formation and reduced NO bioavailability as one causal mechanism. It is still controversial whether intracellular downstream signaling to nitrovasodilator-derived NO is affected as well.

Methods and Results—we therefore studied the effects of 3-day NTG treatment of rats and rabbits on activity and expression of the immediate NO target soluble guanylyl cyclase (sGC) and on the cGMP-activated protein kinase I (cGK-I). Tolerance was induced either by chronic NTG infusion via osmotic minipumps (rats) or by NTG patches (rabbits). Western blot analysis, semiquantitative reverse transcription–polymerase chain reaction, and Northern blot analysis revealed significant and comparable increases in the expression of sGC $\alpha_1$ and $\beta_1$ subunit protein and mRNA. Studies with the oxidative fluorescent dye hydroethidine revealed an increase in superoxide in the endothelium and smooth muscle. Stimulation with NADH increased superoxide signals in both layers. Although cGK-I expression in response to low-dose NTG was not changed, a strong reduction in vasodilator-stimulated phosphoprotein (VASP) serine239 phosphorylation (specific substrate of cGK-I) was observed in tolerant tissue from rats and rabbits. Concomitant in vivo and in vitro treatment with vitamin C improved tolerance, reduced oxidative stress, and improved P-VASP.

Conclusions—we therefore conclude that increased expression of sGC in the setting of tolerance reflects a chronic inhibition rather than an induction of the sGC–cGK-I pathway and may be mediated at least in part by increased vascular superoxide. (Circulation. 2001;103:2188-2194.)

Key Words: protein kinases | vasodilator-stimulated phosphoprotein | nitrate tolerance | hydroethidine | superoxide production

Nitroglycerin (NTG) induces vasorelaxation by releasing nitric oxide (NO). NO, an endothelium-derived relaxing factor (EDRF), activates the target enzyme soluble guanylyl cyclase (sGC) and increases tissue levels of the second messenger cGMP. cGMP activates a cGMP-dependent protein kinase (cGK) that mediates vasorelaxation via phosphorylation of proteins that regulate intracellular Ca$^{2+}$ levels. Although short-term application of NTG exhibits high vasodilator and anti-ischemic efficacy, this activity is rapidly lost on long-term treatment because of the development of tolerance. The mechanisms underlying this phenomenon are likely to be multifactorial and may involve neurohormonal adjustments as well as changes intrinsic to the vasculature itself, such as desensitization of the sGC and/or decreased expression of the cGMP-dependent kinase I (cGK-I). Using lucigenin-derived chemiluminescence, we recently showed that NTG increases vascular superoxide in endothelial but also in smooth muscle cells. Increased vascular superoxide may inactivate NO formed via NTG biotransformation but may also have untoward effects on the downstream NO targets sGC and cGK-I.

The hemoprotein sGC is the predominant intracellular NO receptor in vascular smooth muscle cells (for review see Reference 7). The active enzyme is an obligate heterodimer, which in most mammalian tissues consists of $\alpha_1$ (76- to 82-kDa) and $\beta_1$ (70-kDa) protein subunits. Chronic in vivo...
NTG treatment attenuates the increase in vascular cGMP levels in response to acute challenge with endothelium-derived NO and other nitrovasodilators because of so-called cross-tolerance. Furthermore, in vitro exposure of cells and tissues with high NO concentrations desensitizes sGC to nitrovasodilators, a phenomenon called in vitro tolerance. This led to speculations that sGC desensitization may also occur in vivo tolerance.

The cGK-I is highly expressed in vascular smooth muscle cells. Studies with cGK-I-deficient mice and human cells demonstrated a complete disruption of the NO/cGMP signaling pathway in vascular tissue. Therefore, the activity and/or expression of cGK-I may critically influence NTG-induced vasorelaxation. The vasodilator-stimulated phosphoprotein (VASP) phosphorylation and in particular VASP serine239 phosphorylation (P-VASP) has been shown to be a useful monitor for cGK-I activity in intact cells. VASP, a protein highly expressed in vascular cells, including platelets, endothelial cells, and vascular smooth muscle cells, is phosphorylated at 3 distinct sites (serine157, serine239, and threonine278) by both cGK-I and cAMP-dependent protein kinases with overlapping specificity and efficiency. Experiments with cGK-I-deficient human endothelial cells and platelets established that NO donor- and cGMP-mediated VASP phosphorylation is mediated by the cGK-I. On the basis of this evidence, the present study was designed to examine the effects of in vivo NTG tolerance on sGC expression, as well as on downstream targets of sGC, cGK-I, and its substrate VASP. The oxidative fluorescent dye hydroethidine was used to determine the precise localization (endothelium versus smooth muscle) of superoxide production. To elucidate a role for superoxide in modulating P-VASP, animals were treated concomitantly with the antioxidant vitamin C.

Methods
Animal Model, In Vivo Nitrate Tolerance
Male Wistar rats (weight 250 to 300 g) and New Zealand White rabbits (weight 3 to 4 kg) were studied. Tolerance was induced with miniosmotic Alzet pumps or NTG patches (release rate 0.5 mg/h), leading to NTG delivery rates of 10 and 3.0 mg·kg⁻¹·min⁻¹, respectively). Because high concentrations of NO donors have been shown to decrease the expression of cGK-I, NTG was infused in rats in low (10 mg·kg⁻¹·min⁻¹) and high (100 mg·kg⁻¹·min⁻¹) concentrations. To test the effects of oxidative stress on P-VASP, rabbits were treated for 3 days with the antioxidant vitamin C (500 mg/d PO), which was mixed into the drinking water.

Vessel Preparation and Organ Chamber Studies
Aortic rings were suspended in organ chambers, and the NTG dose-response relationship with and without vitamin C (1 mmol/L; 30 minutes) was established as described.

Determination of Vascular Superoxide Production
Lucigenin Assay
Measurement of vascular superoxide was performed with lucigenin (5 μmol/L) as described. To test the effects of in vitro incubations on vascular superoxide production, aortic rings from NTG-treated rabbits were incubated with vitamin C (10 mmol/L; 60 minutes).

Oxidative Fluorescent Microtopography
The oxidative fluorescent dye hydroethidine was used to evaluate the in situ concentration of superoxide as described. In the presence of superoxide, hydroethidine is rapidly oxidized to ethidium bromide (EtBr), where it is trapped by intercalation with DNA. Some rings were incubated with NADH (100 μmol/L; 30 minutes) before being cross sectioned.

RT-PCR of α₁ and β₁ sGC mRNA
RNA extraction from aortic tissue, quantification, and reverse transcription–polymerase chain reaction (RT-PCR) for both sGC subunits and elongation factor II were performed as described.

RNA Blot Hybridization for α₁ and β₁ sGC mRNA
Total aortic RNA was extracted as described above, modified by the single-step RNA isolation method with Trizol reagent (Gibco-BRL).
RNA was extracted from 2 mg of total RNA with oligo(dT) cellulose (Gibco-BRL). One to 2 mg of poly(A) mRNA was fractionated in 1.2% agarose-formaldehyde gels containing EtBr. The RNA was transferred to Porablot nylon membranes (Macherey-Nagel), cross-linked by exposure to UV light, and baked (80°C) for 2 hours. Membranes were hybridized with biotinylated cDNA probes (BioPrime DNA labeling system, Gibco-BRL) recognizing rat sGC α1 and β1 subunits and elongation factor II, the latter being used for verification of equal loading. Positive bands were identified with the Photogene nucleic acid detection system (Gibco-BRL).

**sGC Western Blot**

Western blotting for sGC α1 and β1 subunits was performed as described previously for the β1 subunit. 17

**Determination of sGC Activity**

The activity of sGC was determined by conversion of [α-32P]GTP into [32P]cGMP as described.8

**Detection of cGK-I Expression, cGK-I Activity, and P-VASP**

Aortic tissue was frozen and homogenized in liquid nitrogen. SDS-PAGE and electroblotting was performed as with sGC. The membrane was then divided horizontally at 65 kDa. A polyclonal antibody against cGK-I13 and a mouse monoclonal antibody (16C2) specific for VASP phosphorylated at serine239 11 was used. All data reported here as P-VASP indicate VASP phosphorylation at serine239. A second blot was used to detect VASP expression by use of a phosphorylation-independent monoclonal antibody against VASP (IE273).18 Immunodetections were done with anti-rabbit/mouse secondary antibodies and were visualized as described above. As positive controls for cGK-I or P-VASP, we used 10 ng cGK-I and 10 μg protein of sodium nitroprusside (SNP)–stimulated human platelets, respectively. In separate experiments, aortas from control and NTG-treated rats were incubated with high concentrations of SNP (10 μmol/L), which have been shown to cause maximal relaxation in control and tolerant aortas. This was performed to test whether the cGMP-dependent signaling pathway is still intact in tolerant tissue. To study a role for oxidative stress in the regulation of P-VASP in response to NTG treatment, aortas from tolerant rabbits were treated in vitro with vitamin C (1 mmol/L for 30 minutes).

**Statistical Analysis**

Results are expressed as mean±SEM. The ED50 value for each experiment was obtained by logit transformation. To compare
superoxide production, P-VASP and VASP, cGK-I, sGC expression, and sGC activity in normal and NTG-treated animals, 1-way ANOVA was used. Comparisons of vascular responses were performed with multivariate ANOVA. A Scheffé's post hoc test was used to examine differences between groups when significance was indicated. Probability values of $P<0.05$ were considered significant.

Results

Effects of NTG Treatment on EtBr Fluorescence in Endothelial and Smooth Muscle Cells

NTG treatment increased EtBr fluorescence in the endothelium and smooth muscle, reflecting increased superoxide. Preincubation of aortic segments with NADH markedly increased EtBr fluorescence in the endothelium and media of NTG-treated animals but not in controls (Figure 1).

Effects of In Vivo NTG Treatment on sGC $\alpha_1$ and $\beta_1$ mRNA and Protein Expression in Rat Aorta

Compared with controls, NTG treatment caused a 2-fold increase in the mRNA abundance of sGC $\alpha_1$ and $\beta_1$ as assessed by semiquantitative RT-PCR (Figure 2A) and by nonradioactive Northern blot technique (Figure 2B) ($n=4$; $P<0.05$). The increase in sGC message was accompanied by a comparable increase in sGC $\alpha_1$ and $\beta_1$ protein in rat aorta (Figure 3) as well as in rabbit aorta (data not shown).

Effects of In Vivo NTG Treatment on sGC Activity in Homogenates From Rat Aorta

The SNP-stimulated sGC activity in protein extracts from tolerant aortas was higher than in controls (Figure 4).

Influence of In Vivo NTG Treatment on cGK-I Expression and on the Levels of Basal and Ex Vivo SNP-Induced P-VASP

In vivo NTG treatment caused a substantial drop in P-VASP in rat (Figure 5) as well as in rabbit aorta (Figure 6) without altering cGK-I and VASP expression. Incubation of control and tolerant tissue with high concentrations of SNP caused comparable maximal levels of P-VASP in rat aorta (Figure 5). $\beta$-Tubulin expression did not change in response to NTG treatment (Figure 6).

Influence of In Vivo Low- and High-Dose NTG Treatment on Vascular cGK-I Expression

Infusion of NTG in high (100 $\mu$g · kg$^{-1}$ · min$^{-1}$) but not low (10 $\mu$g · kg$^{-1}$ · min$^{-1}$) concentrations significantly decreased cGK-I expression in rat aorta (Figure 7).

Influence of In Vitro and In Vivo Vitamin C Treatment on NTG Dose-Response Relationship, Vascular Superoxide, and P-VASP

In vitro vitamin C treatment of aortas from in vivo NTG-treated rabbits improved tolerance, reduced vascular super-

Figure 5. A, Effects of NTG infusion on expression of cGK-I and VASP in rat aortas and on phosphorylation of VASP serine 239. Densitometric quantification of Western blots. B, Densitometric quantification of effect of in vitro incubation with SNP on phosphorylation of VASP serine 239 in aortas from control and NTG-treated rats. Data are presented as mean ± SEM of 4 experiments. *$P<0.05$ vs control.

Figure 6. Effects of NTG treatment on expression of cGMP-dependent protein kinase (cGK-I) and VASP in rabbit aortas and on P-VASP. Top, Original blots of control and tolerant rabbit aortas; bottom, densitometric quantification. *$P<0.05$ vs control.
oxide, and increased P-VASP. The effects were even more striking in New Zealand White rabbits treated concomitantly with vitamin C in vivo (Figure 8).

**Discussion**

We demonstrate here for the first time that in vivo treatment with NTG leads to increased expression of the NO target enzyme sGC in 2 different animal models of nitrate tolerance. Downstream signaling of sGC by cGMP-dependent protein kinase (cGK-I), however, was markedly attenuated, as indicated by the pronounced reduction in the basal phosphorylation of the cGK-I substrate VASP. The oxidative fluorescent probe hydroethidine showed increased superoxide in endothelial as well as in smooth muscle cells, which was further increased after stimulation with NADH. Tolerant tissue responded fully to the nitrovasodilator SNP in concentrations that cause maximal relaxation in control and tolerant tissue, with comparable maxima in P-VASP. In vivo as well as in vitro treatment with the antioxidant vitamin C improved vascular relaxations to NTG in NTG-treated rabbits, reduced vascular superoxide, and increased P-VASP, indicating a contribution of oxidative stress to these phenomena. Decreased P-VASP in the presence of increased expression of sGC may indicate that in the setting of tolerance, the NO/cGMP pathway is inhibited rather than activated.

Previously, we and others have shown that treatment of rats and rabbits with NTG leads to a marked attenuation of vasodilator responses to NTG as well as to NO/EDRF-eliciting agonists.4,5 Because in vivo NTG tolerance is associated with decreased basal and NO-induced cGMP level in vascular tissue, Molina et al5 hypothesized that sGC in vascular smooth muscle was desensitized by chronic exposure to NTG. Recent studies have shown that chronic NTG treatment increases superoxide in endothelial as well as in smooth muscle cells4,9 and that removal of endothelium as well as treatment with liposomal superoxide dismutase (SOD) partially but not completely improved NTG-elicited relaxations in tolerant tissue.4 This observation suggests that increased endothelial and/or smooth muscle superoxide production may be an important determinant of in vivo nitrate tolerance.

**Effects of Chronic NTG Treatment on sGC Activity and Expression**

By analyzing the effects of 3-day infusion of rats with NTG on sGC activity in the cytosolic fraction from endothelium-intact aortic tissue homogenate, we made the surprising observation that the SNP-stimulated sGC activity in tolerant aorta was significantly higher than in nontolerant aorta. In direct support of these findings, activation by the NO-independent sGC activator YC-1 (100 μmol/L)19 was also 2-fold higher in tolerant aortic cytosol than in control cytosol (data not shown). The fact that vascular sGC, although “desensitized” in intact tissue, is fully responsive to NO in protein extracts from tolerant tissue suggests that the desensitizing process in intact tissue is not operative in protein extracts. This is in accordance with the hypothesis that in intact tissue, superoxide generated by a membrane-bound NADH oxidase accounts for the reduced bioavailability of NTG-derived NO and may also directly inhibit sGC.19 This oxidase is not active in the protein extract under the present sGC assay conditions, and consequently, activation of sGC by NO is not impaired.

By analyzing the effects of NTG treatment on the expression of sGC in tolerant rat and rabbit aorta, we found a
significant increase in sGC subunit mRNAs and protein. At first glance, the increased sGC expression in NTG tolerance is unexpected. Recently, however, endothelial dysfunction of the aorta in an animal model of chronic ischemic cardiomyopathy was found to be associated with increased vascular superoxide production, depressed vascular cGMP levels, and increased expression of NO synthase III and the sGC β1 subunit. Because cGMP responses were restored by the SOD mimetic Tiron, the authors concluded that the sGC under these conditions was chronically inhibited by increased vascular superoxide production. The coincidence between the 2 animal models (heart failure and nitrate tolerance) with respect to sGC expression is striking and may point to a common mechanism of upregulation of sGC expression.

Effects of Chronic NTG Treatment on the Expression and Activity of cGK-I

To assess whether the downstream target of sGC, cGK-I, was also affected by NTG, we analyzed the expression of this protein by Western blotting. In aortas from NTG-tolerant rats and rabbits, no changes in cGK-I expression were observed. These data appear to be inconsistent with the recent findings from Soff et al. who reported significant downregulation of cGK-I expression in rat vascular smooth muscle in response to in vivo treatment with the organic nitrate ISDN. To induce tolerance in our model, however, the NTG concentration was 10 μg·kg⁻¹·min⁻¹ in rats and 3 to 5 μg·kg⁻¹·min⁻¹ in rabbits. NTG concentrations used in patients range from 0.5 μg·kg⁻¹·min⁻¹ in patients with coronary artery disease and myocardial infarction up to 7 μg·kg⁻¹·min⁻¹ in patients with heart failure. The ISDN concentrations used in the rat model by Soff et al were 34, 68, and 152 μg·kg⁻¹·min⁻¹. Thus, using much lower NTG concentrations, we can induce a high degree of tolerance without alteration of cGK-I expression. With high NTG concentrations, such as 100 μg·kg⁻¹·min⁻¹, however, we found a significant drop in the expression of cGK-I similar to the extent observed by Soff et al. This indicates that decreased expression of cGK-I does not contribute significantly to the development of tolerance/cross-tolerance in our models but may well occur when very high concentrations of NO donors are used.

To reconcile our findings with the reduced nitrovasodilator responsiveness in the setting of NTG tolerance, we assessed the activity of cGK-I in intact aortic tissue from tolerant and nontolerant animals. For this purpose, we studied the phosphorylation of the 46/50-kDa VASP at serine239 to monitor cGK-I activity. VASP is a well-characterized substrate for cGK-I and cAMP-dependent protein kinase (cAK) in platelets, endothelial cells, and vascular smooth muscle cells. Functionally, VASP is a crucial factor involved in the regulation of spatially confined actin polymerization. In platelets, VASP and VASP phosphorylation are known to be involved in basal and vasodilator-induced inhibition of integrin activation and subsequent aggregation. Activation of cAK and cGK can be analyzed by specific monoclonal antibodies directed against differently phosphorylated phospho-VASP forms, because cAK and cGK preferentially phosphorylate VASP at serine157 and serine239, respectively. Experiments with cGK-I–deficient mice established that NO-induced VASP phosphorylation is mediated primarily by the cGK-I.

In the present study, we found significant levels of P-VASP in untreated aortic tissue from rats and rabbits and a striking reduction of P-VASP in aortic tissue from NTG-treated animals compared with untreated controls. The presence of P-VASP in aorta of untreated animals suggests that endogenous cGMP- and/or cAMP-elevating vasodilators (NO and/or prostacyclin) maintain a certain level of P-VASP in the vascular wall. The decrease in P-VASP induced by chronic NTG treatment was not due to decreased availability of this cGK-I substrate, because total VASP expression at the protein level was not different in tolerant and nontolerant aorta. Incubation of control as well as tolerant tissue with the NO donor SNP revealed comparable increases in the phosphorylation of VASP, indicating that the cGMP-signaling pathway downstream of sGC per se is not impaired provided that sufficient NO is available. We have shown previously that cAMP-dependent vasodilation by forskolin is not altered in the setting of nitrate tolerance. Furthermore, expression of cAK- and cAMP-mediated VASP phosphorylation in aortic tissue was not significantly affected by NTG pretreatment (data not shown).

These findings clearly indicate that the NO/cGMP pathway is functionally inhibited in NTG-induced tolerance despite increased expression of NO-sensitive sGC and normal protein levels of both cGK-I and VASP. It appears unlikely that activation of a phospho-VASP phosphatase or an increased activity of a cGK-I inhibitor or of phosphodiestersases could account for decreased VASP phosphorylation. This is supported by the observations that the vascular relaxation in response to direct cGK-I activators such as 8-bromo-cGMP and the extent of maximal SNP-induced P-VASP (Figure 5) are not altered during nitrate tolerance.

Mechanisms Underlying Inhibition of VASP Phosphorylation

By using EtBr fluorescence, we were able to demonstrate (in agreement with previous observations) that NTG increases superoxide production not only in endothelial cells but also in smooth muscle cells. To identify a possible involvement of a superoxide source, we incubated intact vascular tissue with 100 μmol/L NADH as described recently. Using this approach, Guzik et al showed that there is a close correlation between NADH-stimulated superoxide release from intact vessels and from homogenates, suggesting that use of either approach to measure NADH oxidase activity seems to be valid. Stimulation of tolerant but not of control tissue with NADH markedly increased EtBr fluorescence, compatible with the activation of an NADH-driven oxidase. Indeed, we recently identified an NADH-dependent oxidase as a superoxide source in nitrate tolerance as well as decreased activity and/or expression of the CuZn SOD. In vitro studies demonstrated a marked regulation of NO-elicited pulmonary artery relaxation and guanylyl cyclase activation by the NADH oxidase and SOD. Incubation of vascular homogenates with NADH as well as the inhibition of SOD by diethylthiocarbamate strikingly inhibited sGC activity and NO-mediated vasorelaxation, respectively. Thus, these
findings suggest that an NADH-dependent and SOD-sensitive activity in the smooth muscle of tolerant aortic tissue inhibits NO-induced sGC activation to such an extent that cGMP formation is decreased below nontolerant levels, as observed experimentally, despite a higher sGC protein expression compared with nontolerant aorta.

The concept that oxidative stress may, at least in part, be causally involved in tolerance is further strengthened by the demonstration that in vitro incubations with vitamin C as well as in vivo vitamin C treatment partially reversed tolerance, reduced vascular superoxide, and, subsequently improved P-VASP (see Figure 8).

Conclusions
This study demonstrates an impairment of the NO/cGMP signaling cascade during nitrate-induced tolerance at the level of NO-mediated sGC activation. Quantification of P-VASP appears to be a powerful tool to assess the functional integrity of the NO/cGMP effector pathway in vascular tissue. The demonstration of increased superoxide in smooth muscle cells, together with the known inhibitory effects of superoxide on sGC activity, suggests that both the oxidative stress concept of tolerance and the desensitization concept of sGC (as originally proposed by Murad’s group) may explain, at least in part, the nitrate tolerance phenomenon.

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References
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