Explaining the Phenomenon of Nitrate Tolerance

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Abstract—During the last century, nitroglycerin has been the most commonly used antiischemic and antiangiinal agent. Unfortunately, after continuous application, its therapeutic efficacy rapidly vanishes. Neurohormonal activation of vasoconstrictor signals and intravascular volume expansion constitute early counter-regulatory responses (pseudotolerance), whereas long-term treatment induces intrinsic vascular changes, eg, a loss of nitrovasodilator-responsiveness (vascular tolerance). This is caused by increased vascular superoxide production and a supersensitivity to vasoconstrictors secondary to a tonic activation of protein kinase C. NADPH oxidase(s) and uncoupled endothelial nitric oxide synthase have been proposed as superoxide sources. Superoxide and vascular NO rapidly form peroxynitrite, which aggravates tolerance by promoting NO synthase uncoupling and inhibition of soluble guanylyl cyclase and prostacyclin synthase. This oxidative stress concept may explain why radical scavengers and substances, which reduce oxidative stress indirectly, are able to relieve tolerance and endothelial dysfunction. Recent work has defined a new tolerance mechanism, ie, an inhibition of mitochondrial aldehyde dehydrogenase, the enzyme that accomplishes bioactivation of nitroglycerin, and has identified mitochondria as an additional source of reactive oxygen species. Nitroglycerin-induced reactive oxygen species inhibit the bioactivation of nitroglycerin by thiol oxidation of aldehyde dehydrogenase. Both mechanisms, increased oxidative stress and impaired bioactivation of nitroglycerin, can be joined to provide a new concept for nitroglycerin tolerance and cross-tolerance. The consequences of these processes for the nitroglycerin downstream targets soluble guanylyl cyclase, cGMP-dependent protein kinase, cGMP-degrading phosphodiesterases, and toxic side effects contributing to endothelial dysfunction, such as inhibition of prostacyclin synthase, are discussed in this review. (Circ Res. 2005;97:618-628.)

Key Words: oxidative stress • nitroglycerin • nitric oxide • endothelial dysfunction

Nitroglycerin (GTN) has been one of the most widely used antiischemic drugs for more than a century. Given acutely, organic nitrates are excellent agents for the treatment of stable-effort angina, unstable angina, in patients with acute myocardial infarction and in patients with chronic congestive heart failure. The chronic efficacy of nitrates, however, is blunted because of the development of nitrate tolerance. The problem of tolerance has been raised since the first clinical reports of nitrate therapy for hypertension in Bright’s disease. In 1888, Stewart reported a case of GTN tolerance in a man...
who required 20 grains of pure GTN to achieve the same hypotensive effect as induced by the initial dose of 1/100 grain. He later stressed that this was a common problem in clinical practice. The mechanisms underlying this phenomenon are still poorly defined. Nevertheless, recent data indicate that GTN-induced reactive oxygen species (ROS) formation may inhibit the GTN-metabolizing enzyme. This combination of adverse effects may explain the phenomenon of the clinically relevant tolerance and cross-tolerance to endothelium-dependent and -independent nitrovasodilators.

Mechanisms of GTN Activation and Action

How Does GTN Induce Vasodilation?

There is a major consent that the principle mechanism of GTN-induced smooth muscle relaxation is the activation of the intracellular NO receptor enzyme, soluble guanylyl cyclase (sGC), subsequent elevation of the cyclic GMP (cGMP) levels, and activation of cGMP-dependent protein kinases, and/or cyclic nucleotide-gated ion channels. Thus, GTN and other organic nitrates are believed to use the same signaling mechanism as NO generated by NO synthases. However, the precise mechanism by which NO activates vascular sGC is still controversially discussed.

Soluble GC is a heterodimeric hemoprotein consisting of α and β subunits. The enzyme is strongly activated by binding of NO to the ferrous heme-iron, half maximally at about 1 μmol/L. steady-state concentration of NO. GTN requires intracellular (endothelial and/or smooth muscle cells) bioconversion to elicit cGMP formation and vasodilation, and, until recently, NO was regarded as the bioactive metabolite. Following the discovery of endothelium-derived NO, the idea of NO being the active principle of GTN was very attractive and led to the speculation that GTN may replace a compromised endothelial NO production, such as in patients with coronary heart disease. Several studies supported the GTN/NO hypothesis by demonstrating the formation of NO in cells and tissues exposed to GTN, either in vitro or in vivo. However, because in all of these studies, GTN was applied in concentrations far exceeding the therapeutic range, it remained unresolved whether GTN in therapeutically effective low concentrations increases vascular NO levels.

No NO From GTN?

We recently addressed this issue by NO spin trapping and electron paramagnetic resonance (EPR)-detection using colloid iron-diethylthiocarbamate, Fe(DETC). Cyclic GMP formation was indirectly assessed by analyzing the phosphorylation state of the vasodilator-stimulated phosphoprotein (VASP) at Ser239 (P-VASP). VASP is a prominent cGMP-dependent protein kinase (cGK) substrate and a reliable biochemical marker of cGK-I activity. In endothelium-intact vessels, we found that GTN exhibited a striking dissociation (3 log units of concentration difference) between its vascular activity (increase in P-VASP and vasorelaxation, observed with nanomolar concentrations) and its NO-donor properties (increase in NOFe(DETC)₂ formation, observed at micromolar concentrations). This finding was in contrast to the coincidence of NO formation and vascular activity seen with isosorbide dinitrate (ISDN) and calcium ionophore (A23187) in the same vessel type and caused doubt on the GTN/NO hypothesis. In support of this finding, the vasodilator activity of GTN was much less susceptible to inhibition by the NO scavenger carboxy-PTIO than that of established NO donors. These results challenge the widely accepted GTN/NO hypothesis and suggest that at therapeutically effective (nanomolar) concentrations, GTN activates the vascular sGC/cGK-I pathway independently of a biotransformation of NO. Interestingly, however, in the presence of the endothelium GTN applied in micromolar concentrations exhibits lower vasodilator potency, yet higher NO formation than in endothelium-denuded vessels. This finding indicates that metabolism of higher concentrations of GTN to NO, occurs preferentially in endothelial cells. Our findings raise the question how GTN can activate sGC and elicit vasorelaxation independent of EPR-detectable NO formation. An explanation may be that the bioactive GTN metabolite influences the activity of sGC indirectly via modulation of its intracellular localization and/or its interaction with sGC-binding proteins. It is also possible that an sGC-activating factor chemically closely related to NO but not detectable by spin trapping is formed from GTN. This issue is still unresolved.

GTN Downstream Signaling

Following activation of sGC the resulting increase in cGMP triggers further signaling events by activation of protein kinases, cation channels, and cyclic nucleotide-degrading phosphodiesterases. These shape the biological response, ie, inhibition of smooth muscle contraction, of proliferation etc. For details, see page I of the online data supplement available at http://circres.ahajournals.org.

GTN Biotransformation

GTN is metabolized by different pathways, which are linked either to its activation or inactivation. Metabolites generated by the inactivating routes are thought to include inorganic nitrite and nitrate, and glycerol-1,3-dinitrate (1,3-GDN). This metabolism is accomplished by glutathione reductase (GR) and glutathione-S-transferase (GST). Metabolites generated by bioactivation routes include NO, S-nitrosothiols, inorganic nitrite and glycerol-1,2-dinitrate (1,2-GDN). The bioactivation pathway appears to differ between low (therapeutic) and high (pharmacological) concentrations of GTN. As described above, the pathway realized at antiischemic and vasodilating concentrations of GTN (nanomolar range) does not generate measurable amounts of NO. Other characteristic features of the so-called high-potency pathway (Figure 1) not shared by the low-potency pathway include a susceptibility to inhibition by pertussis toxin and by superoxide-generating unspecific inhibitors of sGC such as LY83583 and methylene blue. The high-potency pathway is also prone to a rapid GTN-induced tachyphylaxis.
Differences Between Organic Nitrates

Structurally different organic nitrates exhibit different therapeutic profiles with regard to unwanted reactions and the benefit provided to the patients in specific disease states. This is not only because of different pharmacokinetics, pharmaco-dynamics, dosing regimen, and routes of application, but also because of different bioactivation mechanisms. We recently assessed whether other organic nitrates besides GTN are also bioactivated by ALDH-2 in rat aorta.33 The highly potent organic nitrates esters such as GTN, pentaerythrityl tetrinitrate (PETN), and pentaerythrityl trinitrate (PETriN) were bioactivated by ALDH-2, whereas less potent nitrates such as ISDN, isosorbide-5-mononitrate (ISMN), pentaerythrityl di-nitrate (PEDN), and pentaerythrityl mononitrate (PEMN) were not. The vasodilator potency of the organic nitrates closely correlated to their susceptibility to inhibition of vasodilator responses, cGMP formation and cGK activity (P-VASP formation) by benomyl, their potency to inhibit ALDH-2-dehydrogenase activity in mitochondria from rat heart, and their potency to increase mitochondrial superoxide formation in vitro.33 The group of the highly potent nitrates could be further differentiated by their capability to block ALDH-2-esterase activity. This activity was not affected by ISDN, ISMN, PETN, or its metabolites PETriN, PEDN, PEMN, whereas it was inhibited by benomyl, GTN (500 μmol/L) applied in vitro, peroxynitrite (500 μmol/L), hydrogen peroxide (10 mmol/L), and in aorta from in vivo GTN-tolerant rats. These findings show that GTN specifically inhibits the ALDH-2 esterase activity, whereas PETN and PETriN do not. This difference suggests that the ALDH-2 esterase activity is required for bioactivation of the highly potent organic nitrates and might explain why GTN elicits nitrate tolerance with much higher potency than PETN or the other nitrates.

The concept that different pathways account for bioactivation of highly potent nitrates at low and high concentrations (see under sections Low-Potency Pathway for Bioactivation of GTN and High-Potency Pathway for Bioactivation of GTN; Figure 1) also extends to the organic nitrates with low vasodilator potency, such as ISDN and ISMN. It appears that the di- and mononitrates are not bioactivated by ALDH-233 but by other routes that are effective only with high concentrations of organic nitrates. A favorable candidate for this low-potency biotransformation is cytochrome P450 (Figure 1), which has been shown to accomplish NO formation from ISDN in the liver34 and in human coronary arteries.35

Also with respect to induction of nitrate headache, organic nitrates show remarkable differences. GTN most frequently induces headache, followed by ISDN, whereas ISMN and PETN clearly induce less headache.36,37 These differences may be related to vessel-type selectivity and pharmacokinetics.

Nitrate Tolerance

Nitrate tolerance is a complex phenomenon, which involves neurohormonal counter-regulation, collectively classified as pseudotolerance,23 as well as intrinsic vascular processes, defined as vascular tolerance. GTN-induced desensitization of vasodilator responses to NO donors and endothelium-derived NO is termed cross-tolerance. A typical phenomenon associated with vascular tolerance is the worsening of anginal symptoms compared with pretreatment state after cessation of nitrate therapy, the so-called withdrawal or rebound effect. All of these states have to be discerned from an acute loss of GTN efficacy at intermediate to high concentrations in in vitro experiments, the so-called tachyphylaxis.

Vascular and Systemic Features of Nitrate Tolerance

Role for Neurohormonal Counter Regulation in Nitrate Tolerance?

GTN therapy is associated with activation of neurohormonal vasocostricter forces. This has been demonstrated for intra-

Figure 1. Pathways of organic nitrate bioactivation in vascular cells. The high-potency nitrates (GTN, PETN, PETriN) are bioactivated by mitochondrial ALDH-2 when used in low doses, generating clinically relevant concentrations (<1 μmol/L). The reductase activity converts the organic nitrates to nitrite and the denitrated metabolite (1,2-glyceril dinitrate, PETriN, or its dinitrate PEDN). Nitrite requires further bioactivation by either reduction by the respiratory chain (cytochrome oxidase [CytOx]) or by acidic disproportionation in the intermembrane space (H^+), finally yielding NO or a related species (NO_x), which activate sGC and trigger cGMP signaling via cGK-I. The low-potency nitrates (ISDN, ISMN, GDN, PEDN, and their respective mononitrates glyceryl mononitrate [GMN] and PEMN) are bioactivated by P450 enzyme(s) in the endoplasmic reticulum (smooth ER) directly yielding NO. The latter mechanism also accounts for the high potency nitrates used at high doses.
venous GTN therapy and with GTN patches in patients with coronary artery disease, heart failure, and control subjects. The GTN dose used ranged between 0.3 µg/kg per minute in controls and patients with coronary artery disease and 5 to 7 µg/kg per minute in patients with heart failure. GTN-induced drops in blood pressure cause a baroreflex stimulation leading to a variety of neurohormonal adjustments. These include increases in catecholamine levels and release rates, increases in plasma vasopressin, plasma renin activity (reflecting increased circulating angiotensin II), and aldosterone levels (Figure 2). These changes are not GTN specific and have also been observed during therapy with other vasodilators. The degree of neurohormonal stimulation depends on the dose of GTN.

GTN therapy is also associated with a marked increase in intravascular volume, which may attenuate the preload effect of GTN. A decrease in hematocrit during long-term GTN treatment very likely reflects intravascular volume expansion secondary to a transvascular shift of fluid caused by an alteration in Starling forces and/or aldosterone-mediated salt and water retention. All of these neurohormonal counter-regulatory mechanisms are regarded as pseudotolerance.

Enhanced Sensitivity to Vasoconstriction Contributes to Nitrate Tolerance

A further mechanism contributing to vascular tolerance is the increased sensitivity to receptor-dependent vasoconstrictors (Figure 2). This has been shown in rabbits treated with GTN for a 3-day period in a clinically relevant concentration of 1.5 µg/kg per minute and in rats chronically infused with GTN. Reports from patients with coronary artery disease also indicate that this observation may have clinical significance. Heitzer et al observed that reductions in forearm blood flow in response to intraarterial (brachial artery) angiotensin II and phenylephrine were markedly enhanced in patients pretreated with GTN for a 48-hour period (0.5 µg/kg per minute). These hypercontractile responses could be blocked by concomitant treatment with the angiotensin-converting enzyme inhibitor captopril, suggesting that an activated renin angiotensin system is responsible, in part, for this phenomenon. Therefore, an increase in sensitivity to vasoconstrictors represents a major mechanism responsible for the attenuation of the vasodilator effects of GTN.

In nitrate tolerant rabbits, we could normalize vasoconstrictor responses in vitro by inhibitors of protein kinase C (PKC), an important enzyme for maintenance of agonist-induced smooth muscle contraction. Furthermore, treatment of rats with PKC inhibitors prevented, in parallel, nitrate tolerance and the sensitization to vasoconstrictors. Interestingly, increased sensitivity was not demonstrated for a classical activator of PKC, such as endothelin-1 (ET-1). We speculated that locally produced ET-1 already occupied the receptors. Indeed, vessels from GTN-treated rabbits, in contrast to controls, exhibited strong ET-1 and big-ET-1 immunostaining within the media. Thus, increases in local ET-1 may either downregulate or occupy ET-1 receptors, making them unavailable for activation by exogenous ET-1. In support of these findings, oxidative stress markedly stimulated the expression of ET-1 in cultured endothelial and smooth muscle cells. Because in the setting of tolerance, increased ROS production is observed in all layers of the vascular wall, it is very likely that the increase in ET-1 expression in tolerant tissue is elicited by oxidative stress.
GTN Treatment Induces Endothelial Dysfunction: The Phenomenon of Cross-Tolerance

A phenomenon related to nitratre tolerance is cross-tolerance to the vasodilator action of other organic nitrates, NO donors, and endothelium-derived NO. This has been observed most commonly when GTN was administered chronically in experimental animal models and not when nitratre tolerance was produced in vitro by short-term exposure (1 hour) of isolated vascular segments to high concentrations of GTN (0.55 mmol/L). Endothelial dysfunction (ED) can be observed in humans during prolonged GTN therapy. In large coronary arteries, Caramori et al found that continuous treatment (5 days) with GTN patches leads to enhanced acetylcholine (ACh)-induced paradoxical constriction, instead of endothelium-dependent vasodilation, which was taken as a surrogate parameter for ED. By using strain gauge plethysmography Gori and coworkers showed that chronic (6-day) GTN treatment (0.6 mg/h, GTN patches) resulted in a marked reduction of ACh-infusion–induced dilation. Likewise, the vasoconstriction elicited in control subjects by L-NMMA (NOS inhibitor) infusion, which unmasks a tonic release of NO, was significantly blunted in volunteers treated with GTN. In the lowest concentration, L-NMMA even caused a paradoxical dilation. The authors concluded that GTN treatment reduces basal as well as agonist-stimulated vascular NO bioavailability and that this may, at least in part, be attributable to abnormalities in NOS function, eg, that NOS generates a vasoconstrictor agent. Taken together, we believe that chronic GTN treatment causes ED (Figure 2), which may have important clinical implications, because ED has been shown to be a predictor of adverse long-term outcome in patients with coronary artery disease.

Nitrate Tolerance: Molecular Mechanisms

Does Oxidative Stress Account for Nitrate Tolerance and Cross-Tolerance?

In 1995, we defined a new molecular mechanism accounting for GTN tolerance and cross-tolerance. We then found that aortic segments from 3-day GTN-exposed rabbits were tolerant to the vasodilator action of GTN in vitro and exhibited cross-tolerance to ACh and the sydnonimine SIN-1, confirming previous reports by Murad and coworkers. The removal of the endothelium, however, markedly attenuated tolerance to GTN and cross-tolerance to SIN-1, suggesting a substantial role of the endothelium in mediating tolerance, as recently confirmed by de la Lande et al. We hypothesized that the endothelium is either chronically releasing a vasoconstrictor and/or that NO (or the NO-like bioactive principle of GTN) becomes chemically inactivated before it can stimulate the sGC in vascular smooth muscle. In support of this hypothesis, we found that the superoxide levels in tolerant vessels amounted to approximately twice that in the controls and were normalized by removal of the endothelium. Because diphenylene iodonium acutely inhibited superoxide formation, we suggested a flavin-containing oxidase as a likely superoxide source and detected an increased activity of membrane-bound NADH/NADPH oxidase in tolerant vasculatlar tissue. Similarly, in nitrate tolerant human volunteers and chronically GTN-infused dogs, an increase in platelet NAD(P)H oxidase activity and superoxide formation has been detected. So far, it is not known whether nitrate tolerance increases the expression of subunits critical for NADPH oxidase activity or whether it stimulates an association of cytosolic subunits with the membrane-bound cytochrome b5/p22phox oxidase components.

Subsequently, we demonstrated that GTN treatment stimulates the vascular production of peroxynitrite, an ROS generated from a rapid reaction of NO with superoxide (Figure 2). A stable metabolite of peroxynitrite, nitrotyrosine, is formed by nitration of tyrosine, either free or protein bound. In vitro and in vivo data indicated that GTN treatment increased vascular and urinary nitrotyrosine levels, which can be taken as a semiquantitative indicator of increased peroxynitrite formation. Increased vascular peroxynitrite formation may affect the proper function of NOSIII and thus induce ED by different mechanisms.

Peroxynitrite can oxidize the NOSIII cofactor tetrahydrobiopterin (BH4) to dihydrobiopterin (BH2) via intermediate formation of trihydrobiopterin (BH3) radicals (Figure 2). Provided that dihydrobiopterin reductase activity is not sufficient, the resulting intracellular BH deficiency may lead to dysfunctional NOSIII. Dysfunctional NOSIII can bind and transfer electrons to molecular oxygen, but further reaction with L-arginine is not possible. Consequently, NOSIII becomes uncoupled and releases superoxide (Figure 2). Thus, GTN therapy may switch NOSIII from an NO donor to a superoxide-producing enzyme, which may further increase oxidative stress in vascular tissue in a positive feedback fashion. Indeed, we recently demonstrated increased expression of an uncoupled NOS in an animal model of nitrate tolerance, by showing that an inhibitor of NOS, L-NNA, significantly reduced vascular superoxide production in tolerant vessels. In addition, supplementation of GTN-treated rats with BH4 reversed GTN-induced ED, further indicating that ED induced by chronic GTN treatment is, at least in part, secondary to intracellular depletion of BH4. The clinical relevance of this experimental finding has been recently highlighted. In these studies, the authors could not only demonstrate that GTN-induced ED responded well to treatment with folic acid, a substrate for BH4 synthesis, they also found a large improvement of nitrate tolerance in forearm vessels of healthy volunteers. In support of these findings, recent in vitro studies indicate that folic acid restores NOSIII function by increasing depleted intracellular BH4 levels. Furthermore, the laboratory of Fung addressed the effect of nitrate tolerance in rats (10 μg/min intravenously for 8 hours) on vascular gene expression and observed a marked 53% decrease in GTP-cyclohydrolase I feedback regulatory protein mRNA. This protein controls the rate of BH4 synthesis by GTP cyclohydrolase (Figure 2). Provided that mRNA and protein expression are directly correlated (which we do not know), the rate of BH4 synthesis will be reduced by half in nitrate-tolerant tissue.

Interestingly, exogenously applied L-arginine improved tolerance in rat aorta and humans. An explanation for this unexpected finding may be provided by the observation that...
t-arginine addition can prevent immediate GTN-induced superoxide formation in cultured bovine aortic endothelial cells. However, the significance of this finding for in vivo tolerance is unclear.

GTN (1 hour, 10 μmol/L) has been shown to activate PKC in cultured endothelial cells, as indicated by a transient membrane translocation of PKCa and PKCe. This response was associated with increased tyrosine nitration, which could be blocked by peroxynitrite scavengers (uric acid), superoxide dismutase, Nε-nitro-t-arginine methyl ester, and the PKC inhibitor chelerythrine. The authors concluded that GTN-induced activation of specific PKC isoforms triggers intracellular events leading to NOS uncoupling. Activation of endothelial PKC induces phosphorylation of NOSIII, leading to an inhibition of NO production by the enzyme, all of which may also contribute to GTN-induced ED. Because activation of PKC is induced by superoxide and peroxynitrite, a vicious cycle is set up by GTN, involving mutual activation of PKC, increased ROS production, depletion of intracellular BH4, and uncoupling of NOSIII (Figure 2). The GTN-induced increase in oxidative stress stimulates production of endothelin-1 within endothelial and smooth muscle cells, leading to further PKC activation, which, in turn, may trigger enhanced constrictor responses to almost every receptor-dependent agonist (Figure 2). PKC may also activate NADPH oxidases in the vasculature, contributing to increased vascular superoxide formation.

The finding that the degree of GTN tolerance was similar in NOSIII knock-out and wild-type mice does not disqualify uncoupled NOSIII from the mechanism of GTN tolerance, because neuronal-type NOSI can functionally substitute for NOSIII in NOSIII knock-out mice. It is conceivable that in these mice, vascular NOSI will be uncoupled in the nitrate-tolerant state.

Sage et al showed that nitrate tolerance in patients is causally related to increased superoxide formation and reduced GTN biotransformation in human blood vessels. Rings prepared from the internal mammary artery and saphenous vein of patients treated for 24 hours with GTN (10 μg/min intravenously) before elective bypass surgery were tolerant to GTN, exhibited increased superoxide formation as detected by lucigenin (10 μmol/L) chemiluminescence, and generated 40% less 1,2-dinitroglycerin, the metabolite derived from bioactivation of GTN (Figure 1). They failed, however, to demonstrate cross-tolerance to endothelium-dependent (A23187) and -independent (sodium nitroprusside) vasodilators. Also, an acute 3-fold increase in vascular superoxide production by exposure to the superoxide dismutase (SOD) inhibitor diethyldithiocarbamate did not modify the GTN dose-response relationship. Therefore, the authors concluded that impaired GTN biotransformation more likely accounts for tolerance than vascular superoxide formation and that the endothelial function is preserved in the tolerant state.

In contrast, using higher GTN doses (35 μg/min intravenously) and longer treatment periods (48 hours), we could demonstrate endothelial dysfunction in the artery mammaria and arteria radialis in patients undergoing coronary bypass surgery and markedly increased superoxide production in these vessels, confirming our previous findings in patients with stable coronary artery disease. The failure of ED development in the study by Sage et al also contrasts to the results from Gori et al and Caramori et al. The discrepant findings are very likely to be explained by the different dose and duration of GTN application. After 1 day of GTN exposure, pseudotolerance may still prevail over vascular tolerance.

Further support for the oxidative stress concept was provided by the demonstration that nitrate tolerance achieved by 7-day GTN treatment (0.6 mg/h) of healthy volunteers increased the plasma levels of cytotoxic aldehydes and isoprostanones, which are considered as sensitive markers for free radical–induced lipid peroxidation. Similarly, in isolated platelets of healthy volunteers exposed to 0.4 mg/h GTN for 3 days, McGrath et al detected increased esterified 8-epi-PGF2α, a marker of oxidative stress and COX activation. To address the role of mitochondrial oxidative stress in nitrate tolerance, we recently assessed the effect of GTN on wild-type versus heterozygous Mn-SOD deficient (Mn-SOD−/−) mice. Mn-SOD is the mitochondrial SOD isoform. We detected increased ROS formation and decreased ALDH-2 activity in isolated mitochondria from Mn-SOD-deficient mice. The aorta of these animals exhibited an increased sensitivity for development of nitrate tolerance on acute challenges of isolated vessels with GTN and on chronic GTN infusion of these mice. These findings suggest that increased mitochondrial oxidative stress could be a decisive component in nitrate tolerance development.

**Effect of GTN-Induced Superoxide/Peroxynitrite Production on Prostacyclin Synthase**

Recent findings show that prostacyclin synthase is a highly vulnerable target of peroxynitrite and that chronic GTN application in vivo inhibits this enzyme via peroxynitrite-induced tyrosine nitration. Consequently, prostacyclin (PGH2) formation is decreased and prostaglandin H2 (PGH2) formation increased in nitrate tolerance, leading to increased vascular tone. For details, see page 2 of the online data supplement.

**GTN Signaling Targets Hit by Superoxide and Peroxynitrite**

**Effects of GTN Tolerance on sGC Activity and Expression**

In nitrate tolerance, not only reduced bioactivation of GTN will decrease stimulation of vascular sGC, but GTN tolerance-induced superoxide and peroxynitrite may also directly interfere with nitrovasodilator action at the level of sGC (Figure 2). Both superoxide and peroxynitrite are potent direct inhibitors of NO-sensitive sGC, and reduced basal and NO-stimulated sGC activities were detected in peroxynitrite-exposed cells and vascular tissues. We also found that in the absence of glutathione very low concentrations (<1 μmol/L) of peroxynitrite nearly abolished NO-dependent, as well as NO-independent (YC-1), activation of the purified enzyme (A.M., unpublished results, 2000). Interestingly, exposure of the purified enzyme to a fully inhibitory concentration of peroxynitrite (1 μmol/L) did not induce
formation of immunodetectable nitrotyrosine on sGC subunits, suggesting that this inhibition is not accomplished by nitration of tyrosine residues. Similarly, in homogenates from GTN-tolerant rabbits, sGC was not detected by a 3-nitrotyrosine antibody (A.M., unpublished results, 2000), indicating that sGC is not tyrosine nitrated in nitrate tolerance. Provided that peroxinitrite and superoxide formation is sufficiently high in nitrate tolerant tissues, tonic inhibition of sGC may contribute to cross-tolerance to other nitrovasodilators, NO-donors and endothelium-dependent agonists, as observed previously\(^5\) (Figure 2). Our (unexpected) recent observation that expression of sGC subunits \(\alpha_1\) and \(\beta_1\) was increased in nitrate-tolerant vascular tissue\(^6\) may be interpreted as a biological counter-regulatory mechanism compensating partially for lower sGC activity and cGMP formation.

Effect of GTN-Induced Superoxide/Peroxynitrite Production on the Activity and Expression of the cGMP-Dependent Protein Kinase

How does GTN-induced stimulation of superoxide/peroxynitrite production influence intracellular cGMP downstream signaling? Previous studies with mice deficient in cGMP-dependent protein kinase (cGK-I) highlighted the crucial role of this enzyme in mediating cGMP-stimulated vasodilation.\(^9\) The phosphorylation of the vasodilator stimulated phosphoprotein (VASP) at Ser239 (P-VASP) has been shown to be useful for monitoring cGK-I activity in vascular tissue. Changes in vascular P-VASP levels are closely related to endothelial function and oxidative stress,\(^21\) suggesting that P-VASP can be used as a novel, biochemical surrogate parameter for vascular NO-bioavailability and/or efficiency of cGMP downstream signaling.\(^22\) We could not detect any changes in cGK-I expression in aortas from GTN-tolerant rats and rabbits compared with controls, but a striking reduction of phosphorylated VASP when compared with untreated controls was detected.

To address specifically the role of oxidative stress in inhibiting the NO-signaling, the phosphorylation level of VASP in GTN tolerant vascular tissue was quantified in response to in vitro and in vivo treatment with the superoxide/peroxynitrite scavenger vitamin C, ebselen, and uric acid. We could demonstrate that all of these antioxidants markedly restored P-VASP, decreased superoxide levels and nitrotyrosine formation to control, and, accordingly, restored GTN sensitivity in vessels from GTN-tolerant animals.\(^86,87\) These observations were recently confirmed with studies testing the effects of long-term GTN infusion on cGMP/cGK-I signaling in patients with coronary artery disease.\(^22\) For details on beneficial effects of antioxidants on the development of nitrate tolerance, see pages 15 to 18 in the online data supplement.

Effect of GTN Therapy on the Activity and Expression of Cyclic Nucleotide-Metabolizing Phosphodiesterases

Because vasorelaxation to GTN is mediated by increased cGMP formation, this response is sensitive to regulation by cGMP-metabolizing phosphodiesterases (PDE) (Figure 2). It is, therefore, not surprising that several previous studies showed a relief from nitrate tolerance in vitro and in vivo by application of PDE inhibitors such as the PDE5A1-specific compound zaprinast.\(^9,91\) These findings fostered the speculation that nitrate tolerance might be based on an increase in cGMP-degrading PDE activity and/or expression in the vascular smooth muscle. Indeed, in chronically GTN-infused rats, we detected a marked increase in the activity and expression of the Ca\(^{2+}\)/calmodulin–dependent PDE1A1 isoform, whereas expression of Ca\(^{2+}\)-independent PDE5A1 was not appreciably altered.\(^92\) Accordingly, the PDE1-specific inhibitor vinpocetine partially restored nitrate sensitivity of the tolerant vasculature. The increase in the expression of this enzyme would, at least in part, explain the decreased responsiveness of the tolerant vasculature to GTN and to endothelium-dependent vasodilators (cross-tolerance), the decreased activity of the cGMP-dependent kinase/NO signaling, as assessed by P-VASP, and the increase in sensitivity to intracellular Ca\(^{2+}\)-eliciting vasoconstrictors observed in animal and in human studies. Interestingly, angiotensin II infusion had a similar effect on PDE1A1 activity and expression as GTN infusion,\(^92\) which would explain why angiotensin-converting enzyme inhibitors positively influence GTN-induced hypersensitivity to vasoconstrictors and GTN-induced tolerance and cross-tolerance. A recent study addressing alterations in vascular gene expression in nitrate tolerant state (8 hours of GTN infusion in rats) detected a 1.8-fold increased expression of cGMP-stimulated cAMP-metabolizing PDE2A2 mRNA in tolerant rat aorta, whereas an increase in PDE1A1 mRNA was not reported.\(^93\) This may provide a hint that cross-talk between cAMP and cGMP metabolism might also be affected in nitrate tolerance. On the other hand, the gene expression data of the study by Wang and Kim were collected at different stages of nitrate tolerance (8 hours versus 3 days), and tolerance was induced by different GTN doses (10 \(\mu\)g/min intravenously versus 2.5 to 3 \(\mu\)g/min subcutaneously), which may well explain the different findings.

Mechanisms Underlying Tolerance: Impaired Biotransformation Versus Oxidative Stress Concept

Within the last decades, several concepts concerning the mechanisms underlying nitrate tolerance have been intensively discussed. One favorite hypothesis originating from the earlier work by Needleman et al\(^4\) and later modified by others was that impaired GTN bioactivation leads to decreased GTN sensitivity in the tolerant vasculature. The other concept, discovered by us,\(^52\) claimed increased oxidative stress and reduced NO bioavailability as the mechanism underlying nitrate tolerance. Pros and cons have frequently been raised over the years, without conclusion. For instance, impaired bioactivation of GTN may not explain associated phenomena such as endothelial dysfunction, increased sensitivity to vasoconstrictors, and/or increased vascular superoxide production.

A clue to these apparently contradicting concepts appeared recently by the identification of ALDH-2 as the enzyme responsible for GTN bioactivation\(^3,33,95–97\) (see preceding paragraph). Chen et al showed in in vitro studies that
tolerance-inducing high concentrations of GTN (0.3 mmol/L, 30 minutes) inhibited ALDH-2 dehydrogenase activity and GTN biotransformation to 1,2-glyceryl dinitrate (GDN) (GTN reductase activity), frequently taken as a monitor for bioactivation of GTN. Similarly, in vitro high-dose GTN exposure attenuated cGMP increases in response to acute GTN challenge. We assessed whether inhibition of ALDH-2 also accounts for nitrate tolerance in vivo. We found that the aortas from tolerant (3-day GTN-infused) rats exhibited reduced GTN vasodilator responses, but in contrast to non-tolerant controls, in vitro treatment of in vivo tolerant aortic rings with ALDH-2 inhibitors did not affect the GTN concentration-response curve. Total ALDH activity in vascular homogenates and in mitochondria isolated from tolerant rat aorta and heart was reduced by more than 50% compared with controls. Daidzin, a more specific ALDH-2 inhibitor, caused a similar decrease in isolated control tissues and mitochondria. Furthermore, acute exposure of isolated mitochondria to a higher concentration of GTN (5 to 500 μmol/L) decreased ALDH activity to a similar extent, and, in addition, stimulated mitochondrial superoxide formation. Inhibition of complex III by antimycin A similarly elicited superoxide formation and inhibition of ALDH activity. In addition, mitochondria isolated from tolerant animals generated ROS at ≈50% higher rate than control mitochondria, and this was entirely blocked by acute addition of DTT, uric acid, or ebselen. The effects of nitrate tolerance on classical ALDH activity were mirrored by a similar reduction of GTN reductase activity, as detected by formation of 1,2-GDN. In conclusion, these findings show that in vivo nitrate tolerance is caused by inhibition of ALDH-2 and suggest that GTN metabolism triggers superoxide production within mitochondria (Figure 2). These data actually confirm previous observations published by Needleman and Hunter showing that incubation of isolated heart mitochondria with high concentrations of nitrates induced swelling of mitochondria, stimulated oxygen consumption, and uncoupled oxidative phosphorylation, consistent with a mitochondrial source of nitrate-elicited ROS. Regardless of the exact mechanism by which GTN stimulates ROS production (eg, premature release of partially reduced oxygen from complex IV, accumulation of toxic aldehydes, initiation of lipid peroxidation, depolarization of mitochondrial membrane potential, mitochondrial swelling, etc), loss of ALDH-2 activity should cause GTN to accumulate in mitochondria and, thus, amplify the effect (Figure 2). Chen et al proposed that oxidation of essential thiol groups in the active site of the enzyme underlies the molecular mechanism of tolerance, as supported by our study. However, it is not known whether the artificial substrate GTN and/or ROS cause inhibition of ALDH-2. In studies with purified yeast ALDH, we found that GTN, superoxide, and peroxynitrite were all capable of directly inhibiting the enzyme (A.D., unpublished observations, 2004). These findings support the idea that oxidative stress may contribute directly to mechanism-based tolerance, either by oxidative inhibition of ALDH-2 and/or perhaps by depleting essential cofactors required for reactivation of oxidized ALDH-2, such as lipoic acid. Irrespective of the exact sequence of events, incubation of tolerant tissue with various disulfide-reducing agents (dithiothreitol) and antioxidants (vitamin C) completely restored vascular ALDH activity and simultaneously normalized mitochondrial ROS production. Inasmuch as nitrate tolerance may underlie the increases in cardiac morbidity seen with chronic nitrate use (based on metaanalysis), these observations may have therapeutic implications. Earlier observations that ALDH dehydrogenase activity is reduced in nitrate tolerant patients provide support for the clinical relevance of this mechanism.

Do All Nitrates Induce Tolerance? Although tolerance development occurs with all nitrates, depending on dose and duration of treatment, PETN is a remarkable exception. This organic nitrate exhibits considerably less tolerance-inducing activity than all of the others. This property could be related to the unique antioxidative defense protein-inducing properties of PETN and its metabolites. For details, see page 3 of the online data supplement.

Old and New Strategies to Prevent the Development of Tolerance and Cross-Tolerance Several clinical approaches to prevent nitrate tolerance were tested in the past; however, none of them has yet been accepted as a gold standard. For details, see page 4 of online data supplement.

Summary and Clinical Implications In summary, continuous systemic therapy with organic nitrates induces tolerance and endothelial dysfunction in patients with coronary artery disease and even in healthy controls. Mechanisms contributing to this phenomenon may be a nitrate-induced stimulation of vascular (mitochondrial) superoxide and/or peroxynitrite production and the ensuing inhibition of ALDH-2, leading to impaired biotransformation of GTN. Several studies indicate that chronic GTN treatment worsens endothelial function and a recent (ex-post) metaanalysis indicates that nitrates may worsen prognosis in patients with ischemic heart disease. Further studies are required, however, to understand the precise nature of mechanisms underlying GTN-induced endothelial dysfunction to develop strategies to prevent these GTN-induced side effects (see online data supplement, pages 8 to 18), although there are probably significant differences between organic nitrates with respect to bioactivation pathways, induction of ROS formation and tolerance development. More generally, mitochondrial injury and the ensuing oxidative stress unify concepts of tolerance and cross-tolerance and provide a molecular rationale for the range of agents that seemingly prevent the development of nitrate tolerance. It is likely that agents ameliorating oxidative stress (eg, angiotensin converting enzyme inhibitors, angiotensin II type 1 receptor blockers, statins, l-arginine, BH₃, ascorbate) will restore sensitivity to GTN, but it remains to be seen whether they are able to prevent inhibition of ALDH-2 during prolonged GTN treatment. Likewise, it will be of interest to see whether ebselen and uric acid, which lessen mitochondrial ROS production and preserve ALDH-2 activity in vitro, can also do so in vivo and whether this confers protection from tolerance.
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References


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Supplementary information to “Explaining the phenomenon of nitrate tolerance” by Munzel et al

GTN downstream signaling.

Pre-eminent cGMP effectors are cGMP-dependent protein kinases (cGK-I and cGK-II) and cyclic nucleotide-gated (CNG) ion channels. The mechanisms whereby cGK-I, the isoform expressed in vascular smooth muscle, lowers agonist-induced contractile tone have been reviewed in detail recently\(^1\) and are only shortly summarized here. Principal vasodilating mechanisms include a cGMP/cGK-I-induced decrease of agonist-induced intracellular free Ca\(^{2+}\) levels in smooth muscle cells and a desensitization of contractile elements to Ca\(^{2+}\). The former is accomplished by several independent processes: A cGK-I-induced phosphorylation of the IP\(_3\)-receptor-associated cGK-substrate (IRAG) prevents IP\(_3\)-dependent release of Ca\(^{2+}\) from the sarcoplasmatic reticulum\(^2\). Secondly, cGK-I reduces extracellular Ca\(^{2+}\) entry through potential-operated channels by phosphorylation of the Ca\(^{2+}\)-activated (Maxi)K\(^+\)-channel (BK\(_{\text{Ca}}\)), thereby increasing the open probability of this channel and hyperpolarizing the smooth muscle cell membrane\(^3\). Furthermore, cGK-I may accelerate Ca\(^{2+}\) uptake into the SR by phospholamban-dependent and/or -independent activation of the SR Ca\(^{2+}\)-ATPase (SERCA)\(^4\) thereby also inhibiting capacitative Ca\(^{2+}\) entry via Ca\(^{2+}\)-store-operated Ca\(^{2+}\) channels in smooth muscle and endothelial cells\(^5\). In addition, contractile elements are desensitized to Ca\(^{2+}\) by cGK-I-dependent activation of myosin light chain (MLC) phosphatase\(^6\), and by inhibition of RhoA/Rho-kinase-dependent intracellular contractile signaling\(^7\), both resulting in dephosphorylation of MLC.

The endothelium might influence the mechanism of GTN-induced
vasorelaxation\textsuperscript{8}. In endothelium intact coronary arteries GTN relaxation was partially inhibited by iberiotoxin (specific inhibitor of BK\textsubscript{Ca}-channels), though not in endothelium denuded vessels. Thus, GTN relaxes endothelium-intact vessels also by activating endothelial and/or smooth muscle cell BK\textsubscript{Ca}-channels. This effect appeared to be mediated by sGC/cGMP, since relaxations to GTN were sensitive to inhibition by the sGC inhibitor NS2028\textsuperscript{9}, and NS2028-insensitive relaxation was not blocked by iberiotoxin.

GTN can also affect vascular eicosanoid production\textsuperscript{10}, which lead to the speculation that GTN might affect vascular tone by this mechanism, too. Indeed, GTN was shown to activate cyclooxygenase 1 (COX-1)\textsuperscript{11}, leading to enhanced PGI\textsubscript{2}-formation in cultured endothelial cells and vascular tissue\textsuperscript{12,13}.

**The low potency pathway for bioactivation of GTN**

The low potency pathway leads to formation of measurable amounts of NO in vascular tissues in vivo\textsuperscript{14} and in vitro\textsuperscript{15} (print version figure 1). Therefore, NO is a vasoactive principle of of GTN applied in higher concentrations. Previous studies, which focused on the identification of enzymes and/or low molecular mass factors which could generate NO from GTN, identified cysteine, N-acetyl-cysteine and thiosalicylic acid\textsuperscript{16,17}, as well as deoxyhemoglobin, deoxymyoglobin\textsuperscript{18}, cytochrome P\textsubscript{450} (CYP)\textsuperscript{19} and xanthine oxidase\textsuperscript{20}. Since the non-enzymatic reaction of GTN with thiols requires high concentrations (mM) of these thiols as well as GTN (µM range) this reaction may lack physiological significance. While NO formation by desoxy-hemoproteins and xanthine oxidase will be confined to tissues of low oxygen tension (hypoxia), the CYP pathway is likely to account for NO formation from GTN accumulating in high concentrations in liver, lung and kidney\textsuperscript{14}- Induction of hepatic
CYP isoforms by glucocorticoids and other agents increases NO and cGMP formation from GTN in these tissues\textsuperscript{21}, whereas 3d infusion of GTN decreases hepatic CYP expression\textsuperscript{22,23}. A similar effect was not observed with sodium nitroprusside infusion, indicating that the decrease of CYP expression may specifically depend on GTN metabolism by CYP.

In addition to its function in hepatic metabolism of GTN CYP is also a favorable candidate for catalyzing NO formation from GTN in vascular tissues exposed to high concentrations of GTN (figure 1 print version). Different isoforms of CYP have been shown to account for NO release from 10 µM GTN in isolated blood vessels of human\textsuperscript{24} and animal origin, the most active CYP isoform in rats being CYP1A2\textsuperscript{25}. Induction of vascular CYP1A2 by 5d treatment of rats with i.p. acetone strongly increased NO release from GTN by isolated veins, abdominal arteries and thoracic aorta, whereas down-regulation of CYP1A2 by 48 h GTN infusion decreased vascular NO formation\textsuperscript{25}.

**The high potency pathway for bioactivation of GTN**

More recently Chen et al. suggested that the mitochondrial isoform of aldehyde dehydrogenase (ALDH-2) is responsible for bioactivation of GTN (figure 1 print version)\textsuperscript{26}. The isolated enzyme generates nitrite (NO\textsubscript{2}\textsuperscript{-}) and glycerol-1,2-dinitrate from GTN, and the reaction is accelerated by NAD\textsuperscript{+} probably by an allosteric action. Unspecific inhibitors of this enzyme (disulfiram, cyanamide, chloral hydrate) and high substrate concentrations (acetaldehyde) attenuated the vasorelaxing, cGMP-eliciting and blood pressure lowering activity of GTN in rats, and inhibited the organic nitrate reductase activity of ALDH-2. Since the enzyme is enriched in the mitochondria isolated mitochondria can mediate activation of isolated sGC by GTN in
a NAD⁺-dependent fashion, and this activation was acutely prevented by daidzin, a highly specific inhibitor of ALDH-2. Using isolated rat aortic rings we recently demonstrated a marked attenuation of the GTN vasodilator potency following incubation with acetaldehyde and choral hydrate, as previously observed in rabbit aortic rings, as well as with benomyl and daidzin. In addition, activation of cGK-I (as assessed by P-VASP) and vasodilation by GTN were markedly inhibited by the ALDH-2 inhibitor benomyl (10µM), whereas benomyl did not modify SNP- or ACh-induced phosphorylation of VASP and vasorelaxation.

We also showed that treatment of RAW 264.7 macrophages with GTN or exposure to benomyl or daidzin reduced GTN bioactivation (1,2-GDN formation). These results confirmed the observations by Chen et al. and pointed to a specific role of ALDH-2 in the cGMP-mediated GTN induced vasorelaxation. By depleting endothelial cells of functional mitochondria (so-called ρ₀ cells) by means of 5 d exposure to ethidiumbromide we could show that GTN-stimulated increases in cGMP were markedly attenuated by this treatment. Interestingly, inhibition of ALDH-2 by benomyl did not completely abolish the vasodilator and cGK-I stimulating activity of GTN. The concentration-response curve was shifted to the right and with higher concentrations of GTN a maximal relaxant response was still achieved. This finding supports our hypothesis of two independent pathways accounting for bioactivation of GTN, were only the high potency pathway is dependent on ALDH-2 activity (figure 1 print version). Ais mechanism has been proposed to explain the nitrate reductase activity of ALDH-2. The enzyme also exhibits nitrate-esterase activity which forms a hypothetic thionitrate-intermediate from GTN in its active center, with concomitant release of glycerol-1,2-dinitrate. The intermediate then spontaneously stabilizes by nucleophilic attack of an adjacent second cystein thiol-group under formation of a
disulfide bridge and with nitrite as the leaving group. The inactive thiol-oxidized enzyme can be reduced and re-activated by thiol donors like dithiothreitol and 2-mercaptoethanol. Our recent observations suggest that mitochondrial lipoic acid may function as the natural reducing agent (A. Daiber, unpublished observations). Chen et al. suggested that NO could form from NO₂⁻ either via intermediacy of nitrous acid (HNO₂), or by catalysis of components of the mitochondrial respiratory chain, like cytochrome c oxidase (figure 1 print version). NO formation from therapeutic concentrations of GTN (< 1 µM) by this enzyme or isolated mitochondria has, however, not been demonstrated yet. The important role of ALDH-2 for NTG bioactivation was further substantiated by recent studies. Zhang et al. showed that GTN-induced increases in coronary blood flow in chronically instrumented dogs were completely abolished using the ALDH-2-inhibitor cyanamide. In principle this concept is a revival of the Needleman “thiol theory” which already suggested an interaction of organic nitrates with the mitochondria (swelling and increased oxygen uptake) as well as a depletion of mitochondrial thiol pools.

**Effect of GTN-induced superoxide/peroxynitrite production on prostacyclin synthase.**

GTN induced production of peroxynitrite may also adversely affect the activity of the PGI₂-synthase (PGI-S) (figure 2 print version). Recent studies have shown that PGI-S is a preferential nitration target of peroxynitrite. More importantly, tyrosine nitration of PGI-S presumably at tyrosine 430 resulted in an almost complete inhibition of the enzyme activity leading to decreased PGI₂ formation. Since the non-metabolized PGH₂ can activate the TxA₂/PGH₂ receptor of vascular smooth muscle cells and thereby counteract GTN-mediated vasodilation, peroxynitrite can be
considered as a mediator of endothelial dysfunction as well as of "nitrate tolerance" at
the level of the smooth muscle. Our recent data obtained in nitrate tolerant rats and
rabbits go along with this concept. GTN treatment increased the luminol-derived
chemiluminescence signal in rat and rabbit aorta, which was effectively inhibited by
peroxynitrite quenchers such as uric acid as well as by ebselen, compatible with
increased vascular peroxynitrite formation. Western blots of 3-nitrotyrosine
immunoprecipitates exposed to a polyclonal antibody directed against PGI-S
detected a single 52 kDa protein band and revealed a marked increase in the PGI-S
signal from tolerant aortas compared to controls, while total PGI-S expression was
not modified by GTN tolerance. The immunoprecipitation results were in
accordance with immunohistochemical stainings of control and tolerant aorta with
PGI-S and 3-nitrotyrosine antibodies showing increased nitration of PGI-S within the
endothelium of tolerant vessel sections. As a functional consequence of tyrosine
nitration of PGI-S, the conversion of [14C]-PGH₂ into [14C]-PGI₂ (measured by its
stable metabolite 6-keto-PGF₁α) was markedly inhibited. We also observed a shift to
increased PGE₂ formation in tolerant tissue. These findings suggest that tyrosine
nitration accounts for the observed inhibition of the activity of the enzyme in the
setting of nitrate tolerance. The functional inhibition of PGI-S activity in tolerant tissue
was indirectly confirmed by experiments using U51605, the dual blocker of PGI-S
and TXA₂-S, which has previously been shown to mimic the effects of authentic
peroxynitrite on in vitro vasorelaxation. U51605 shifted the concentration-response
relationship for GTN significantly to the right. This finding was interpreted as
indication for accumulation of non-metabolized PGH₂, which is able to elicit
vasoconstriction via activation of the TXA₂/PGH₂-receptor of the vascular smooth
muscle. In contrast, incubation of tolerant tissue with U51605 failed to modify the
remaining vasodilator responses to GTN (which were already right-shifted), because tolerance and U51605 use the same mediator to counteract vasorelaxation, i.e., PGH₂.

Do all nitrates induce tolerance?

A number of studies have shown that clinical signs of tolerance also develop with chronic administration of ISDN and ISMN (studies before 1990 reviewed in³⁷), indicating that tolerance development is uniform to all organic nitrates if applied in clinically effective dosage for longer periods of time. A remarkable exception is pentaerythritol tetranitrate (PETN)³⁸. In contrast to other long-acting nitrates, PETN induces tolerance-free vasodilation in humans³⁹,⁴⁰ and prevents endothelial dysfunction as well as atherogenesis in cholesterol-fed rabbits⁴¹. It was shown that PETN possesses antioxidant properties, which could explain its specific pharmacological profile ⁴⁰. In contrast to all other organic nitrates, PETN and its metabolite pentaerythritol trinitrate (PETriN) induce the antioxidant defense protein heme oxygenase-1 (HO-1), also known as a chaperone, heat shock protein 32 (hsp32), and increase the formation of the antioxidant molecule bilirubin and the (weak) vasodilator carbon monoxide (CO)⁴². In addition, PETN and PETriN increase the expression of another antioxidant protein, ferritin, via the HO-1-dependent release of free iron from endogenous heme sources⁴³. All together, these defense mechanisms protect endothelial cells from hydrogen peroxide-induced toxicity, and might explain the previously observed anti-atherogenic activity of PETN ⁴¹,⁴⁴. In addition, we could show that PETN and PETriN in contrast to GTN did not affect the nitrate esterase activity of ALDH-2, and did not elicit ROS formation in isolated
arteries and mitochondria, adding a further mechanism to explain the lack of
tolerance development to PETN\textsuperscript{28}.

However, with regard to the clinical use of PETN there is an ongoing debate
that an anti-anginally effective dose was not applied in those clinical studies where
administration of PETN in patients with angina did not generate nitrate tolerance.
Indeed, there are studies arguing in both directions, e.g., lack of tolerance
development vs. lack of anti-anginal efficacy because of insufficient dosing\textsuperscript{45,46}.
However, this debate tends to neglect the influence of very special pharmacokinetics
and pharmacodynamics of PETN, the mode of application, the parameters chosen for
identifying anti-anginal efficacy and tolerance, and the patient group selected. Today
PETN is used only as a peroral long-acting nitrate, with a slow onset of action.
Therefore, it is not suitable for acutely relieving anginal pain. However, with
continuous therapy by 80 mg PETN applied 8 hourly, the incidence of anginal attacks
in patients with confirmed angina pectoris and the consumption of nitroglycerin spray
is significantly reduced, even after 4 weeks of continuous therapy\textsuperscript{47}. In a more recent
randomized, double-blinded, cross-over and placebo-controlled clinical study with 15
men with stable angina\textsuperscript{48}, 100 mg PETN p.o. after 2 h exhibited similar anti-anginal
efficacy as 80 mg ISDN and 15 mg NTG (slow release), but loss of efficacy after 6 h
was more rapid with PETN than with the other nitrates. To conclude, these and other
studies suggest that PETN clearly exhibits and maintains anti-anginal activity after
acute and chronic therapy. However, compared to other long-acting nitrates, the
duration of action after single oral application is shorter. Therefore, the current dosing
regimen requires 3 x 80 mg daily in order to achieve continuous anti-anginal
protection. Finally, a recent multi-center clinical study demonstrated the non-
inferiority of PETN vs. ISDN with even partial superiority of the tetranitrate\textsuperscript{49}.
Old and new strategies to prevent the development of tolerance and cross-tolerance.

The most widely accepted approach to prevent the tolerance phenomenon has been a nitrate free interval. This procedure, however, bears several risks. Since oxidative stress is important for tolerance and cross-tolerance, antioxidants or drugs, which are able to reduce oxidative stress within vascular tissue are able to positively influence both phenomena. Recent studies in patients with CAD and heart failure indeed demonstrated that the development of tolerance is beneficially influenced by vitamin C, vitamin E as well as by ACE-inhibitors and statins 50. Most likely sources of ROS in the setting of tolerance are the mitochondria (see printed part), an uncoupled NOS III (see printed part) and NADPH oxidases which are activated by the RAAS (AT II, DAG, PKC).

Nitrate free interval:

Several groups demonstrated that tolerance to the hemodynamic effects of GTN can be avoided using a nitrate free interval or eccentric dosing approaches 51-53. A potential problem related to a nitrate free interval may be the development of rebound ischemia. Cessation of chronic GTN-treatment in experimental animals was not able to normalize endothelial dysfunction and hypersensitivity to vasoconstrictors. During the nitrate free interval the frequency of angina symptoms as well as of silent angina was significantly increased. By treating patients intermittently with GTN patches for a 5 d period Azevedo et al. have shown that this kind of regimen may prevent the development of tolerance 54. On the other hand, acute nitrate withdrawal increased the coronary vasoconstrictor responses to ACh, suggesting that the
rebound phenomenon may be secondary to the development of endothelial dysfunction. Similar data have been obtained with animal experiments where a nitrate free interval improved tolerance, but failed to correct completely endothelial dysfunction and even enhanced supersensitivity to vasoconstrictors such as serotonin and phenylephrine. These data clearly indicate that GTN-induced endothelial dysfunction cannot be prevented by a nitrate free interval. These earlier findings can now be rationalized on the basis of nitrate-induced mitochondrial superoxide formation and inhibition of ALDH-2, which occur in endothelial cells as well as in smooth muscle cells. Endothelial cells may be even more susceptible to GTN-induced damage than smooth muscle cells, since due to their orientation to the vessel lumen they are exposed to higher concentrations of GTN than the smooth muscle cells. A further detrimental effect experienced primarily by the endothelium is the loss of extracellular SOD activity in nitrate tolerant state. As a consequence of reduced ecSOD activity, plasma levels of important antioxidants like α- and β-carotene are reduced, exacerbating the susceptibility of endothelial cells to oxidative damage. Moreover, this leads to formation of toxic carotenoid cleavage products. These products inhibit mitochondrial respiration and increase mitochondrial oxidative stress, thus contributing to nitrate tolerance.

**Nitrate free interval and the rebound phenomenon:**

As pointed out before, one of the most attractive approaches to prevent tolerance development is a 8-12 h nitrate free interval with the disadvantage of lacking protection during this period. Another potential problem can be the development of rebound ischemia. In patients with stable angina Freedman et al. observed an increase in the duration of silent ischemia compared to patients treated
with placebo\textsuperscript{58}. These data go along with reports demonstrating decreased angina threshold after patch removal in smaller\textsuperscript{59} and multicenter trials (so called TIDES II trial)\textsuperscript{60}. Experimental studies provided some insight into the underlying mechanism\textsuperscript{61}. Treatment of rabbits for 3 d with GTN increased vasoconstrictor sensitivity of the tolerant vasculature, induced tolerance, endothelial dysfunction and increased production of reactive oxygen species. Importantly, increased superoxide production and endothelial dysfunction were almost completely prevented by a nitrate free interval, while the supersensitivity to vasoconstrictors persisted\textsuperscript{61}. Thus, the observed increased ACh-induced constriction in the coronary circulation in patients treated intermittently with GTN most likely represents increased vasoconstrictor sensitivity of the smooth muscle to ACh, rather than an attenuation of endothelium-dependent NO-release in response to acute ACh challenges\textsuperscript{54}. More recent clinical trials failed to demonstrate rebound phenomena in patients with CAD treated with nitrates\textsuperscript{62,63}, which may be explained at least in part by the concomitant treatment with an ACE-Inhibitors\textsuperscript{64,65} or beta receptor blockers\textsuperscript{66}.

**Sulfhydryl group donors:**

Studies in non-tolerant and tolerant animals and humans reported a potentiation of GTN bioactivity by sulfhydryl donors N-acetylcysteine\textsuperscript{51,67,68} and L-methionine\textsuperscript{69-71}, but this finding was interpreted by direct non-enzymatic interaction of certain thiols with GTN. The recent findings that oxidation of critical cysteiny1 residues of ALDH-2 may account for nitrate tolerance may now better explain the beneficial effects of SH-group donors on tolerance, since thiol-reducing agents such as dithiothreitol or mercaptoethanol are able to fully restore impaired nitrate reductase activity of the ALDH-2 in vitro. It remains to be shown whether thiol donors act by the
same mechanism in GTN tolerant patients. With respect to our recent observations (involvement of lipoic acid in the reduction of inactivated ALDH-2) a co-therapy with lipoic acid could be of clinical interest.

**Angiotensin converting enzyme inhibitors and AT-1 receptor blockers:**

As discussed previously, vasodilator therapy with GTN is associated with a transient activation of neuro-hormonal vasoconstrictor forces. These counter-regulatory mechanisms may offset the direct vascular effects of GTN within 1 day and together with sodium and water retention may counterbalance the venodilator effects of this drug. Since activation of the RAAS plays an important role in tolerance development, co-treatment with an angiotensin converting enzyme inhibitor or an angiotensin II receptor antagonist should prevent tolerance. However the clinical data concerning the efficacy of ACE-inhibitors in preventing tolerance are contradictory. While Berkenboom et al. 72 and Mehra et al.73 showed that long-term treatment with ramipril prevented nitrate tolerance to GTN and ISDN, respectively, Daka et al. found no improvement of GTN tolerance in patients treated with GTN plus captopril compared to GTN alone74. Studying normal subjects, Katz et al found that tolerance to the venous forearm circulation was prevented by concomitant administration of captopril or enalapril 75, suggesting that the SH-group moiety in ACE-inhibitors is not responsible for the positive effects with respect to tolerance. Similarly, Muiesan et al. showed that the combination therapy of benazepril with transdermal GTN induced a significant increase in exercise duration 22 h post-dosing, while GTN given alone was no longer effective at this time point 76.

Uri Elkayam et al. studied the effects of high dose transdermal GTN (50 and 100 mg respectively) in patients pretreated with an ACE-inhibitor during a three
month period and observed that the combination therapy markedly improved exercise time in response to GTN administration, but not in response to placebo treatment. In addition, GTN significantly decreased end-diastolic and end-systolic dimensions of the left ventricle and augmented fractional shortening indicating that nitrates may represent an important adjunctive therapy in patients pretreated with an ACE-inhibitor.

Although it is difficult to explain the different efficacy of various ACE-inhibitors in these particular studies in preventing or reversing tolerance it is quite possible that the use of higher concentrations of ACE-inhibitors may have influenced the different outcome of these studies. By using a well characterized animal model of nitrate tolerance, we demonstrated that high doses of enalapril (1 mg/kg) prevented the development of tolerance in large coronary arteries and the rebound constriction following cessation of GTN long-term therapy. Low concentrations of enalapril (0.2 mg/kg/day) failed to prevent tolerance and rebound and also failed to increase plasma renin activity for a 24 h period, although this concentration had already a marked effect on angiotensin I pressor responses. This may indicate that a persistent inhibition of angiotensin II formation might be a prerequisite for the observed beneficial effects of high dose enalapril on tolerance development and rebound responses.

Several experimental studies have demonstrated that nitrate tolerance in animal models at the resistance and conductance vessel level was markedly attenuated using AT1-receptor blocker such as losartan. Importantly, these studies also showed that the improvement of tolerance was also accomplished by giving SOD mimetics such as manganese-containing porphyrins, and was also associated with a reduction of oxidative stress in vascular tissue. A recent study
from patients with coronary spastic angina seems to confirm these preclinical observations\textsuperscript{80}. A total of 64 patients were treated with trans-dermal GTN (10mg/d) for a total of 3d. Of these 21 patients were concomitantly treated with an AT1 receptor blocker candesartan (8mg/d). By measuring the brachial artery diameter with ultrasound, the authors found that tolerance to GTN therapy was completely abolished by co-treatment with the AT1 receptor blocker\textsuperscript{80}. In addition, GTN-induced increases in plasma thioredoxin levels (a marker of oxidative stress), were completely inhibited by candesartan, indicating that neuro-hormonal activation during GTN therapy and the subsequent increases in circulating angiotensin II levels may contribute at least in part to the phenomenon of nitrate tolerance via increases in oxidative stress. However, these beneficial effects of sartan therapy on nitrate tolerance in human were questioned by two negative studies. Treatment of healthy volunteers with 75 mg losartan daily for one week could not prevent vascular and hemodynamic tolerance to continuous NTG therapy\textsuperscript{81}. In patients with stable angina pectoris 100 mg losartan daily together with 20 mg transdermal NTG could not prevent tolerance development to the anti-ischemic effect of NTG after 4 weeks of treatment\textsuperscript{82}. A weakness of the latter study was that the exercise test was stopped by most patients due to physical exhaustion, not because of angina, therefore it did not assess the anti-anginal effect of NTG therapy. The discrepant findings between these studies may be explained by the different study groups: patients with spastic angina\textsuperscript{80}, unstable angina\textsuperscript{83}, stable angina\textsuperscript{82}, and healthy volunteers\textsuperscript{81}, and the different functional tests for verifying tolerance development. Clearly, further studies are required to clarify this issue.

\textbf{Hydralazine:}
Favorable interactions between hydralazine and nitrates have been demonstrated in the Veterans Heart Failure Trials (V-HeFT)\(^8\) and in the African-American Heart Failure Trial (A-HeFT)\(^5\). This particular combination has been shown to have beneficial effects on left ventricular function, exercise capacity and most notably in survival in a large patient population with severe heart failure. Hydralazine has also been shown to prevent the development of GTN tolerance in both experimental animals and in humans with congestive heart failure\(^6\). Hydralazine is a strong arteriolar dilator and stimulates reflex increases in vasoconstrictor stimuli including circulating catecholamines and plasma renin activity (reflecting increased circulating angiotensin II levels). This would seem, upon first inspection, to worsen rather than improve tolerance by enhancing the neuro-hormonal counter-regulatory adjustments to the nitrate. An explanation for this apparent paradox may be provided by our finding that a combination therapy of rats with hydralazine and GTN completely prevented the GTN induced increase in vascular superoxide production and tolerance\(^6\). Further, acute addition of hydralazine in clinically relevant concentrations to segments of aorta from control and GTN-treated animals markedly reduced vascular superoxide steady state levels. Hydralazine was only effective when administered in vivo or incubated with intact rings, but had no effect when added to the vascular homogenates. One explanation for this finding is that the effect of hydralazine requires the intact cell to exert its effect, possibility via its hyperpolarizing properties. This possibility is strengthened by the fact that another hyperpolarizing agent, the \(K_{ATP}\) channel activator pinacidil also markedly inhibited vascular superoxide production. In performing relaxation studies we found that tolerance to GTN and cross-tolerance to SIN-1 were completely normalized by hydralazine treatment.
Considering the importance of ALDH-2, is there a relationship to mitochondrial membrane potential and superoxide formation? It appears so. The mitochondrial membrane potential controls mitochondrial matrix volume and intermembrane space, and this in turn controls mitochondrial metabolism. Shrinking of the matrix and increased intermembrane space leads to uncoupling of electron flow and increased mitochondrial superoxide formation.

Several recent studies suggest that hydralazine may be also a potent direct free radical scavenger, and these antioxidant properties of hydralazine were linked to alterations in vascular gene expression. Leiro and colleagues demonstrated an inhibitory effect of hydralazine on inducible NOS/COX-2 gene and protein expression in rat peritoneal macrophages. Hydralazine at 0.1-10 mM inhibited both extracellular and intracellular ROS production by inflammatory macrophages, by a ROS-scavenging mechanism probably affecting superoxide generation by xanthine oxidase and NADPH oxidase. Knowles and coworkers tested whether hypoxia-inducible factor (HIF)-regulating proline hydroxylase might be a target of hydralazine. They found that hydralazine inhibited prolyl hydroxylase domain (PHD) activity and induced nonhydroxylated HIF-1α, taken as evidence for HIF stabilization specifically by inhibition of PHD enzyme activity. Consequently, hydralazine induced a rapid and transient expression of HIF-1α and downstream targets of HIF (endothelin-1, adrenomedullin, heme oxygenase 1, and vascular endothelial growth factor (VEGF)) in endothelial and smooth muscle cells and induced endothelial cell-specific proliferation. In experimental animals hydralazine induced HIF-1α and VEGF protein in tissue extracts and elevated plasma VEGF levels. Thus, hydralazine initiates a pro-angiogenic phenotype and might be beneficial in ischemic disease.
According to preliminary studies in our laboratory, hydralazine is a scavenger of superoxide and an excellent quencher of peroxynitrite-derived free radicals.

**Antioxidants:**

The demonstration of increased superoxide formation in endothelial and smooth muscle cells in GTN tolerance suggests that treatment with antioxidants may prevent this phenomenon. Indeed, studies published by Eberhard Bassenge's group and others demonstrated that concomitant treatment with vitamin C and SOD mimetics such as manganese-porphyrine preserved the sensitivity of the vasculature to organic nitrates. In chronically instrumented dogs, vitamin C completely prevented the development of nitrate tolerance. Recent data also indicate that the antioxidant vitamin E prevents GTN tolerance in forearm veins in humans and restores the depressed cGMP-response of tolerant platelets. In addition, concomitant treatment of patients with congestive heart failure with vitamin C and GTN completely prevented the development of hemodynamic tolerance.

**Carvedilol**

A promising therapeutic approach to prevent nitrate tolerance may be provided by the 3rd generation β-blockers with antioxidant properties, like carvedilol. Watanabe et al. analyzed the effect of carvedilol (10 mg twice a day) and arotinolol (a β-blocker without antioxidant properties) on nitrate tolerance in 24 patients with untreated hypertension. Carvedilol maintained the increase in forearm blood flow (FBF) after sublingual administration of 0.3 mg NTG, whereas this response was lost in the placebo and arotinolol group after 3 days application of a 20 mg/24 h GTN tape. In a similar study the authors observed that carvedilol (2.5 mg per day)
prevented development of nitrate tolerance in patients with chronic heart failure treated for 6 days with 10 mg/24 h GTN tape, whereas metoprolol (30 mg once a day) and doxazosin (0.5 mg once a day) were ineffective. These results indicate that carvedilol may prevent nitrate tolerance in patients with chronic heart failure during continuous therapy with GTN.

In a recent study the effects of 5 d co-infusion of GTN, either with a carvedilol metabolite (BM920228) with antioxidant properties, or with vitamin C (Vit-C) on various hemodynamic parameters were analyzed in chronically instrumented dogs. Co-infusion of either antioxidant prevented development of vascular nitrate tolerance (loss of coronary dilation). In addition, in vitro BM920228 exhibited $O_2^-$ radical scavenging activity similar to Vit-C, and superoxide dismutase (SOD). This finding suggests that the anti-oxidative activity of carvedilol may be based on the direct scavenging of superoxide radicals and presumably other ROS.

**Protein kinase C antagonists:**

As described in the printed part), an activation of protein kinase C and increased sensitivity to vasoconstrictors contributes to nitrate tolerance via. Consistent with this mechanism, in vivo treatment of rats with the protein kinase C antagonists N-benzoyl-staurosporine not only prevented increased constrictions of isolated vessels to catecholamines and thromboxane, but also the development of nitrate tolerance. This observation may suggest that activation of one or several isoforms of protein kinase C within the vasculature may specifically impair the GTN biotransformation process, via promotion of oxidative stress. There are, however, no patient studies with PKC antagonists available.
Statins

In a recent study in normocholesterolemic rats long-term (5 w) treatment with HMG-coenzyme A-inhibitors pravastatin and atorvastatin prevented nitrate tolerance induced by GTN s.c. injections (50 mg/kg/d) for the last three days\textsuperscript{50}. Also, GTN-induced increase in superoxide formation in thoracic aorta was prevented by statin treatment. The protective effect of the statins on GTN-induced relaxant responses and superoxide formation was abolished, when the rats received L-NAME (100 mg/kg/d) concomitantly with GTN. Statin treatment alone increased basal cGMP levels, in accordance with earlier findings that statins improve NOS III activation, presumably via stimulation of Akt phosphorylation\textsuperscript{103}, but do not alter the expression of the enzyme\textsuperscript{50}. The authors concluded that one mechanism whereby statins counteract nitrate tolerance is accomplished by preventing uncoupling of NOS. According to these authors a second mechanism is related to statin-induced downregulation of NADPH oxidases, as shown by Wassmann et al.\textsuperscript{104}, since they observed an acute loss of the protective effect of statins after addition of NADPH to the organ baths. Interestingly, simvastatin pretreatment (1 h) of isolated rat neonatal cardiac myocytes was shown to prevent the hydrogen peroxide-induced depolarization of the mitochondrial membrane potential\textsuperscript{105}. This effect was abolished by L-NAME and 5-hydroxydecanoate, a blocker of ATP-sensitive K\textsuperscript{+} channels. The authors concluded that the statin protected the cardiomyocytes from oxidative injury by acute activation of NOS III via phosphorylation of Akt\textsuperscript{103} thereby increasing bioavailability of NO. NO in turn could activate the (hypothetical) mitochondrial K\textsubscript{ATP} channel, thus counteracting membrane depolarization, uncoupling, superoxide generation and apoptosis. Further studies will have to show by which mechanism statins prevent nitrate tolerance. However, it is evident that the mitochondria are a
common target of GTN and statins.
Cited literature:


89. Leiro JM, Alvarez E, Arranz JA, Cano E, Orallo F. Antioxidant activity and inhibitory effects of hydralazine on inducible NOS/COX-2 gene and protein


