Nitrate Therapy: New Aspects Concerning Molecular Action and Tolerance
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*Circulation* 2011;123;2132-2144
DOI: 10.1161/CIRCULATIONAHA.110.981407
Circulation is published by the American Heart Association. 7272 Greenville Avenue, Dallas, TX 75214
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Nitrate Therapy
New Aspects Concerning Molecular Action and Tolerance

Thomas Münzel, MD; Andreas Daiber, PhD; Tommaso Gori, MD, PhD

Although the short-term vasodilatory properties of organic nitrates are potent and well known, a number of vascular and extravascular changes have been shown to compromise their hemodynamic effects on long-term administration. Among these changes, systemic phenomena such as neurohormonal activation and intravascular volume expansion as well as specific vascular changes such as increased vascular superoxide (O₂⁻) production, increased sensitivity to vasoconstrictors, and decreased responsiveness to nitric oxide (NO) donors have long been identified as playing a role. Several hypotheses have been proposed to explain these abnormalities, and over the last 15 years, our groups have focused on the concept that an inappropriate production of reactive oxygen species (ROS), an impairment in the scavenging of these mediators (Figure 1), or both might have a crucial mechanistic importance in all these modifications. Independently of the role of ROS in tolerance, the possibility that nitrate-induced oxidative stress might affect patients’ prognosis has important implications, and the observation that long-term therapy with most of the drugs in this class causes endothelial dysfunction, the prognostic significance of which is well accepted in patients with coronary artery disease, hypertension, and heart failure, should not be taken as an academic curiosity.

Beyond these as-yet insufficiently investigated prognostic implications, recognition of the role of ROS suggests that a number of interventions thought to interfere with the vascular redox balance might also retard or prevent the development of nitrate tolerance and nitrate-induced side effects. Evidence that therapy with angiotensin-converting enzyme (ACE) inhibitors, angiotensin-1 receptor blockers, certain β-blockers, statins, and vitamins such as folic acid or vitamin C beneficially influence nitrate tolerance and glyceryl trinitrate (GTN)-induced endothelial dysfunction suggests that nitrate therapy might have profoundly different implications since these therapies have become diffusely available. In addition, the recognition of important differences in the mechanisms triggered by different nitrates should also be attributed clinical relevance, and evidence suggests that, rather than the generic nitrate tolerance, more specific expressions (GTN tolerance, isosorbide mononitrate [ISMN] tolerance, etc) should be used. This review summarizes the current concepts underlying tolerance and endothelial dysfunction in response to long-term therapy with different nitrates and addresses the question of whether the use of these drugs remains indicated despite these side effects.

The Hemodynamic Effects of Nitrates
Venous capacitance vessels, large and medium-sized coronary arteries, and collateral vessels are most sensitive to GTN, whereas coronary and peripheral arterioles with a diameter <100 μm are relatively more resistant. In the setting of stable angina, the preferential venodilatation induced by antiischemic doses of GTN results in venous pooling and preload reduction, yielding reduced left ventricular filling pressure and wall tension. As a result of these changes, myocardial workload and oxygen demand decrease (Figure 2). Beyond this effect, the clinical benefit of organic nitrates in the setting of acute coronary syndromes is thought to depend primarily on the dilatation of epicardial coronaries and coronary collaterals, improving perfusion and oxygen delivery to subendocardial regions by increasing total coronary conductance. Furthermore, because the tone and caliber of myocardial resistance vessels are almost unaffected, coronary steal phenomena and reflex tachycardia are in general avoided. In summary, the mismatch between oxygen demand and supply in ischemic regions is rapidly relieved on short-term administration of organic nitrates.

Through the same hemodynamic effects, organic nitrates also markedly improve left ventricular function in patients with congestive heart failure, decreasing right atrial pressure with a redistribution of blood from the central circulation into larger capacitance veins, a reduction in impedance to the left ventricle ejection owing to an increase in compliance of the arterial vasculature, and a reduction in the magnitude, frequency, and velocity of reflected waves in the arterial circulation. The resulting increase in cardiac output reduces left ventricular end-diastolic pressure and wall tension, thereby shifting the stroke-volume/left ventricular end-diastolic pressure relationship from a negative to a positive slope.

Antiaggregant Effects of Organic Nitrates
The effect of nitrates on platelet aggregation and fibrinolysis might also have clinical importance particularly in the setting of acute coronary syndromes in which vascular thrombosis determines low-flow ischemia. Glyceryl trinitrate reduces...
platelet aggregability ex vivo in healthy volunteers and to a lesser extent\(^{10–14}\) (a situation that parallels the reduced vasodilator potency of GTN observed in these clinical conditions\(^{15}\)) in patients with insulin resistance, congestive heart failure, or coronary artery disease. Whether the antiaggregant effects of nitrates have an additional clinical impact on the therapy of coronary artery disease, particularly in light of the introduction and systematic use of targeted antiplatelet agents such as aspirin, thienopyridines, or glycoprotein IIb IIIa inhibitors, is unclear.

**Mechanistic Insight**

The principal mechanism underlying the hemodynamic effects of nitrates is believed to be the activation of the intracellular NO receptor enzyme soluble guanylyl cyclase, leading to increased bioavailability of cGMP and activation of cGMP-dependent protein kinases and/or cyclic nucleotide-gated ion channels (reviewed elsewhere\(^{16}\)). The mechanism of GTN-induced inhibition of platelet aggregation is probably more complex in that it might involve both cGMP-dependent and -independent cGMP pathways\(^{17–20}\). Whatever the mechanism, it appears to be (like the hemodynamic effects [see below]) redox dependent because antioxidants restored GTN responsiveness in heart failure and coronary artery disease patients.\(^{21}\) After the discovery of endogenous (endothelium-derived) NO and the recognition of its importance in controlling vascular homeostasis,\(^{22}\) the concept that nitrates may act as NO donors led to the speculation that these drugs might also be able to compensate for the compromised endothelial function typical of patients with cardiovascular disease. Evidence presented below suggests that this is not the case.

**No Nitric Oxide From Glyceryl Trinitrate?**

Evidence demonstrates that NO per se is not the mediator of the hemodynamic effects of GTN. Using spin trapping techniques, we found a striking dissociation between the vascular activity and NO donor properties\(^{23}\) for GTN compared with ISMN and isosorbide dinitrate (ISDN). Likewise, Nunez et al\(^ {24}\) reported a similar discrepancy between the hemodynamic effects of GTN administration and NO release, suggesting that the action of GTN is unrelated to its bioconversion to NO. In another study, only suprapharmacological doses of short-term oral GTN evoked a measurable NO

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**Figure 1.** Free radical biochemistry. The radical nitric oxide reacts with superoxide to form the highly reactive intermediate peroxynitrite. Superoxide is dismutated by superoxide dismutase (SOD), leading to the formation of hydrogen peroxide (H\(_2\)O\(_2\)) and molecular oxygen (O\(_2\)).

**Figure 2.** Antianginal effects of acutely administered glyceryl trinitrate (GTN).
release. At the time of these observations, however, the mechanism of GTN biotransformation and its relationship with tolerance remained obscure. Evidence consonant with the hypothesis that the mediator is different among different nitrate molecules in this class was also provided by a recent study demonstrating that so-called NO donors such as GTN and pentaerythrityl tetranitrate (PETN) have different effects on myocardial gene expression. Treatment with GTN significantly changed the expression of 532 genes; PETN treatment changed the expression of 1215 genes. Interestingly, only 68 genes were significantly changed by both compounds, raising doubt that a common vasoactive molecule, such as nitric oxide is released from both drugs. Adapted from Pautz et al.26

**Aldehyde Dehydrogenase, the Enzyme That Metabolizes Alcohol, Also Bioactivates Glyceryl Trinitrate**

Glyceryl trinitrate is metabolized by at least 2 different pathways (Figure 4). When administered in high doses (low-affinity pathway), GTN is metabolized by several enzymes, including glutathione-S-transferases, xanthine oxidoreductase, and the cytochrome P450. Beyond this low-affinity pathway, the relevance of which for clinically used dosages remains unclear, a high-affinity pathway was identified in 2002 by Chen et al.,27 who suggested that the mitochondrial aldehyde dehydrogenase (ALDH-2), the enzyme responsible for the catabolism of alcohol, could also have a role in the bioactivation of GTN. Subsequent studies confirmed a marked attenuation of GTN-induced activation of the cGMP-dependent cascade and vasodilator potency after incubation with ALDH-2 inhibitors28,29 and extended these results to PETN; in contrast, ISMN and ISDN were unaffected by ALDH-2 inhibition (or genetic deletion).30,31

The precise active metabolite formed during this process remains obscure; several hypotheses have been proposed. Whatever the nature of this chemical species, these findings found rapid human translation. For instance, incubation of human vessels with an ALDH-2 inhibitor recapitulated the abnormalities associated with the development of tolerance,32 (Figure 5) and a loss-of-function mutation of ALDH-2, particularly frequent in Eastern Asia and Eastern Europe was found to be associated with impaired GTN metabolism and reduced responsiveness to GTN.33

**Nitrate Tolerance**

The clinical introduction of organic nitrates at the end of the 19th century was soon followed by the observation that the hemodynamic and clinical effects of GTN, ISMN, and ISDN invariably wane upon continuous therapy. In the setting of coronary artery disease, nitrate tolerance has been demonstrated as the loss of effects on treadmill walking time and time of onset of angina. In congestive heart failure, it has been described as the loss of hemodynamic effect of the administered nitrate,34 and in hypertension, it is evident as the rapid loss of the hypotensive effects of these drugs. Rather controversial data have been reported for the antiplatelet effects of GTN. A study in dogs showed that tolerance is associated with a paradoxical activation of platelets,35 and another report showed that prior exposure to GTN, even in very low doses, induces tolerance to the antiaggregatory effects of the drug.36 In contrast, other studies in both rats and humans have shown that platelet responsiveness is preserved despite hemodynamic tolerance.37,38 Another issue is the so-called nitrate resistance, ie, the reduced effectiveness of organic nitrates in the setting of cardiovascular disease, which limits nitrate effectiveness independently of prior nitrate use. For instance, McVeigh et
al\textsuperscript{19} reported reduced hemodynamic effects in diabetic patients and (as mentioned above) that GTN-induced inhibition of platelet aggregability is blunted in patients with coronary artery disease or diabetes. To date, it remains unclear whether these different forms of reduced responsiveness to nitrates share common mechanisms (eg, dysfunction of downstream NO signaling pathways) or should rather be considered 2 distinct entities. If the understanding of the mechanism of nitrate bioactivation has proved to be more complex than initially thought, the mechanism of the development of nitrate tolerance is likely even more complex in that it involves neurohormonal counterregulation, expansion of plasma volume (collectively classified as pseudotolerance), and intrinsic vascular processes, defined as true tolerance (the Table). Beyond the loss of the vasodilatory action of nitrates, a typical phenomenon associated with these changes is the worsening of anginal symptoms caused by the withdrawal of nitrate therapy, the so-called rebound effect.\textsuperscript{40}

### Nitrate Pseudotolerance

The vasodilation evoked by intravenous, oral, and transdermal nitrate therapy causes the release of catecholamines\textsuperscript{41} and plasma vasopressin\textsuperscript{11,42} and increases plasma renin activity\textsuperscript{41,42} and aldosterone levels.\textsuperscript{41,42} Such activation of neurohormonal vasoconstrictor forces has been demonstrated in patients with coronary artery disease, patients with heart failure,\textsuperscript{43} and healthy subjects.\textsuperscript{41} In line with these data, long-term continuous transdermal GTN therapy has been associated with altered autonomic neural function, including impaired baroreflex activity and prevalence of sympathetic to parasympathetic tone in the regulation of heart rate.\textsuperscript{44} In addition, in both animal and human studies, long-term therapy with organic nitrates was associated with increased sensitivity to receptor-dependent vasoconstrictors.

#### Table. Hypotheses Proposed to Explain the Development of Nitrate Tolerance

<table>
<thead>
<tr>
<th>Pseudotolerance</th>
<th>Vascular tolerance</th>
</tr>
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<tbody>
<tr>
<td>Activation of the renin-angiotensin-aldosterone system</td>
<td>Impaired GTN biotransformation</td>
</tr>
<tr>
<td>Increase in circulating catecholamine levels and catecholamine release rates</td>
<td>Increased vascular superoxide production</td>
</tr>
<tr>
<td>Increase in vasopressin levels</td>
<td>Desensitization of the soluble guanylate cyclase</td>
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<tr>
<td>Volume expansion</td>
<td>Increase in phosphodiesterase activity</td>
</tr>
<tr>
<td>Impaired GTN biotransformation</td>
<td>Increased sensitivity to vasoconstrictors</td>
</tr>
<tr>
<td>Increased vascular superoxide production</td>
<td>Increased endothelin expression</td>
</tr>
</tbody>
</table>

GTN indicates glyceryl trinitrate.

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\textsuperscript{19} Münzel et al Nitrate Tolerance
such as serotonin, phenylephrine, angiotensin II, and thromboxane.\textsuperscript{3,45}

A marked increase in intravascular volume, secondary to the transvascular shift of fluid and/or to aldosterone-mediated salt and water retention, has also been observed in patients treated with GTN.\textsuperscript{51,42} Although these changes could attenuate the preload effect of GTN, evidence suggests that these mechanisms are not sufficient to fully explain the loss of nitrate effectiveness. For instance, there is a difference in the time frame of neurohormonal activation, plasma expansion, and development of tolerance\textsuperscript{42}; furthermore, studies testing the effects of diuretics, \(\beta\)-blockers, or ACE inhibitors did not invariably reverse or prevent tolerance. Thus, although the possible prognostic implications of these changes need to be acknowledged, other mechanisms of tolerance and a hypothesis that explained all these changes had to be sought.

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

In 1995, we proposed a new molecular mechanism for GTN tolerance and cross-tolerance. Critical to this concept was the evidence that the bioavailability of ROS in tolerant vessels amounted to about twice that in controls, and that this abnormality was corrected by the addition of liposomal superoxide dismutase, which dismutes \(\text{O}_2^{-}\) to \(\text{H}_2\text{O}_2\) and oxygen (Figure 1). Subsequently, we demonstrated that GTN treatment stimulates the vascular (and particularly endothelial) production of peroxynitrite, a highly reactive intermediate generated from the rapid, diffusion-limited reaction of NO with \(\text{O}_2^{-}\) (Figure 1). Evidence of GTN-induced increased ROS production in humans was then obtained ex vivo in arterial segments and in blood or platelets taken from patients rendered tolerant to GTN.\textsuperscript{19,39,48,49} GTN tolerance was also associated with increased markers of free radical–induced lipid peroxidation such as cytotoxic aldehydes and isoprostanes\textsuperscript{50} and esterified 8-epi-PGF\textsubscript{2\alpha}\textsuperscript{51} and with a mild reduction in the responsiveness to the NO donor sodium nitroprusside in healthy volunteers,\textsuperscript{4} which might also be compatible with ROS-mediated interference with NO signaling. From these findings, we proposed the existence of a unifying hypothesis that, founded on the concept of GTN-induced increased oxidative stress, could be compatible with the multiple different observations associated with long-term nitrate therapy.\textsuperscript{6,7,52}

**Mechanisms Underlying Tolerance: Impaired Biotransformation Versus Oxidative Stress Concept**

Several concepts concerning the implications of the oxidative stress hypothesis of nitrate tolerance have been discussed intensively within the last decades. Importantly, the recognition of the role of a mitochondrial enzyme in the biotransformation of organic nitrates and of a role of mitochondrial oxidative stress in the development of tolerance provided a link between 2 only apparently separate hypotheses (reduced bioactivation versus ROS-mediated NO scavenging or ROS-mediated inactivation of NO signaling). This hypothesis is essentially based on the concept that the oxidation of thiol groups may cause inhibition of several enzymes (including ALDH\textsubscript{2}\textsuperscript{21} and guanylyl cyclase\textsuperscript{53}) and therefore both reduced GTN biotransformation inefficient and NO signal transduction.\textsuperscript{28} In line with this, treatment of tolerant animals with mitochondria-targeted antioxidants completely prevented or reversed GTN tolerance,\textsuperscript{54} and heterozygous knockout of manganese-superoxide dismutase\textsuperscript{7}–markedly aggravated tolerance development in response to GTN.\textsuperscript{55}

These data reconcile the bioactivation and oxidative stress hypotheses and provide an interesting clinical corollary of the original observations published by Needleman and Hunter\textsuperscript{56} showing that incubation with high concentrations of nitrates induced swelling of isolated cardiac mitochondria, stimulated oxygen consumption, and uncoupled oxidative phosphorylation, all data that are consistent with a mitochondrial source of nitrate-elicted ROS. Importantly, however, these considerations apply to GTN tolerance, but most likely not to ISMN or ISDN tolerance, because these drugs do not undergo mitochondrial metabolism.\textsuperscript{31}

Regardless of the exact mechanism by which GTN stimulates mitochondrial ROS production (eg, premature release of partially reduced oxygen from mitochondrial complex I or III, initiation of lipid peroxidation, depolarization of mitochondrial membrane potential, mitochondrial swelling\textsuperscript{57}), these observations support the idea that oxidative stress may directly impair GTN biotransformation, either by oxidative inhibition of ALDH-2 or by depletion of essential repair cofactors such as lipoic acid.\textsuperscript{58} Recent data obtained with purified ALDH-2 provide evidence that ALDH-2 could be a source of GTN-triggered ROS formation.\textsuperscript{59} The pathways leading to GTN tolerance in this hypothesis are summarized in Figure 6.

The implications of ROS formation, however, are not limited to the mitochondrial matrix because ROS leaking into the cytoplasm have been demonstrated to activate a cross-talk with the vascular NADPH oxidase,\textsuperscript{60} resulting in further ROS production and in the formation of the highly reactive peroxynitrite. Although the role of each specific free radical is unclear, ROS and/or reactive nitrogen species such as peroxynitrite in turn may oxidize the endothelial NO synthase (eNOS) cofactor BH\textsubscript{4}; cause tyrosine nitration of the prostacyclin synthase, reducing endothelial prostacyclin formation; and directly inhibit the activity of the soluble guanylyl cyclase and/or other enzymes involved in NO signaling.

The impact of ROS on BH\textsubscript{4} is particularly noteworthy. By oxidizing BH\textsubscript{4}, GTN may cause a phenomenon called eNOS uncoupling, whereby this enzyme, rather than NO, produces superoxide, which may further increase oxidative stress in vascular tissue in a positive feedback fashion.\textsuperscript{61} Although the existence of negative reports needs to be acknowledged,\textsuperscript{62} we recently demonstrated increased expression of an uncoupled NOS in an animal model of nitrate tolerance,\textsuperscript{63} an abnormality that was prevented by supplementation of BH\textsubscript{4}.\textsuperscript{64} It is also important to note that changes in ROS production such as an increase in NADPH oxidase activity and evidence for an uncoupled eNOS, all of which could be corrected by administration of vitamin C, have been observed not only in vascular tissue but also in platelets from GTN-treated volunteers.\textsuperscript{59} These observations are consistent with the hypothesis that nitrate-induced oxidative stress, with impairment of
endogenous NO production, might underlie endothelial dysfunction (Figure 7).4,65–68

Unresolved Issues

Although the above considerations have provided a new view of the pathophysiology of nitrate tolerance leading to a unifying hypothesis of the many abnormalities observed in this setting, it needs to be acknowledged that nitrate tolerance remains a complex phenomenon produced by several concurring mechanisms, many of which remain incompletely understood. In addition, a number of controversial issues remain. Although a ROS-dependent interference with NO signaling pathways is compatible with cross-tolerance to endothelium-dependent and -independent nitrovasodilators such as ISMN and ISDN, other hypotheses based on mechanisms that are independent of the oxidative stress concept (such as desensitization of the soluble guanylate cyclase via S-nitrosylation) have also been proposed.69 Notably, inactivation of ALDH-2 does not explain either tolerance to ISMN and ISDN or the existence of a certain degree of cross-tolerance between GTN and these drugs (the metabolism of which is ALDH-2 independent).25,70,71 In addition, induction of cGMP breakdown via phosphodiesterases is also a possible explanation for GTN tolerance and cross-tolerance.72

Furthermore, the role of ALDH-2 inactivation in GTN tolerance was challenged in an animal study that showed that treatment with inhibitors of ALDH-2 cyanamide or propionaldehyde causes a similar dose-dependent decrease in GTN-induced relaxation in both tolerant and nontolerant aorta.73 In addition, despite showing a markedly reduced GTN-induced vasodilation in carriers of ALDH-2 polymorphisms and after ALDH-2 inhibition, human studies suggested that this enzyme accounts for only a half of the total bioactivation of GTN.33 Challenging the role of ROS-induced abnormalities, Sage et al49 reported an impaired biotransformation of GTN in venous tissue from patients treated with GTN but no cross-resistance to other NO donors and to a calcium ionophore; in this study, although the bioavailability of superoxide anion was increased in arterial segments from the same patients, short-term exposure to oxidative stress did not change GTN responsiveness. Further emphasizing the complexity of the mechanisms underlying GTN tolerance, in another study, folic acid preserved arterial, but not venous, responsiveness to sublingual GTN,74 suggesting the existence of different mechanisms across different vascular beds. Finally, the relative importance of the different free radical species (superoxide anion, hydroxyl radical, peroxynitrite, etc) remains unclear.
Clinical Implications

Therapy With Organic Nitrates Impairs Endothelial Function

A number of lines of evidence show that therapy with most organic nitrates in clinically used doses impairs responsiveness to stimuli for the release of endothelium-derived NO (Figure 7). This phenomenon, also known as endothelial dysfunction, has been observed in animal studies and in humans during prolonged GTN, ISMN, and ISDN therapy. In large coronary arteries, continuous treatment (5 days) with transdermal GTN leads to enhanced acetylcholine-induced paradoxical constriction instead of endothelium-dependent vasodilation.65,75 Evidence of impaired responses to acetylcholine was found in arteries removed from patients undergoing nitrate therapy at the time of bypass surgery,48 and continuous treatment with transdermal GTN resulted in a marked reduction of acetylcholine-induced increases in forearm blood flow in healthy volunteers treated with GTN for 6 days.4 Emphasizing the existence of specific abnormalities at the level of the eNOS, the vasoconstriction elicited in control subjects by the infusion of a specific inhibitor was significantly blunted after GTN therapy. Finally, in the lowest concentration, the eNOS inhibitor even caused a paradoxical dilation, which was interpreted as human in vivo evidence of GTN-induced eNOS uncoupling (and resulting paradoxical production of a vasoconstrictor). In line with this finding, GTN-induced endothelial dysfunction was prevented by folic acid, a compound that facilitates recoupling of eNOS.76

Most important, endothelial dysfunction is not limited to treatment with GTN in tolerance-inducing doses; it was also demonstrated after treatment with intermittent dosing of ISMN.66 Evidence of endothelial dysfunction has been shown on withdrawal of nitrate therapy,65,75,77 an observation that might suggest a role of impaired endothelial homeostasis in the rebound phenomenon. So far, the mechanisms (and the enzymatic superoxide source) involved in the increased oxidative stress in response to long-term ISMN treatment remain obscure. No endothelial dysfunction was observed in response to long-term PETN treatment, which is likely due to the upregulation of the antioxidant enzyme hemeoxygenase-1 (HO-1).67 Compared with equipotent dosages of nicorandil, ISDN (40 mg/d) treatment for 3 months was associated with

Figure 7. Organic nitrates cause endothelial dysfunction. Evidence for the development of endothelial dysfunction in response to nitroglycerin (glyceryl trinitrate [GTN]) in peripheral arterioles (A) and coronary arteries (B) and to isosorbide mononitrate (ISMN) peripheral arteries (C) was reported. Pentaerythritol tetranitrate (PETN; D) caused no endothelial dysfunction in the brachial artery, whereas isosorbide dinitrate (ISDN) treatment did (E). Importantly, substances such as folic acid, which have been shown to cause recoupling of an uncoupled nitric oxide (NO) synthase, and the antioxidant vitamin C were able to improve endothelial dysfunction in patients treated with ISMN and GTN. The mechanisms underlying endothelial dysfunction in response to long-term ISDN therapy have not yet been established. FBF indicates forearm blood flow; C, control; LAD, left anterior descending artery; I/N, ratio of infused to noninfused arm; and Ach, acetylcholine. Adapted from Gori et al,4 Caramori et al,66 Thomas et al,66 Schnorbus et al,67 and Sekiya et al,68 with permission of the publisher. Copyright © 1998, 2001, 2007, American College of Cardiology Foundation.

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a reduction in endothelium-dependent flow-mediated dilatation, an effect similar to that observed with GTN (Figure 7).

In summary, evidence that chronic nitrate treatment causes endothelial dysfunction is now substantial. Given its role as a predictor of adverse long-term outcome in patients with coronary artery disease, it will be important to investigate in the future whether chronic therapy with organic nitrates modifies patients’ prognosis.

Are All Nitrates Homogeneous in the Induction of Tolerance and Endothelial Dysfunction

It is well accepted that tolerance develops in response to long-term continuous administration of ISDN and ISMN (studies before 1990 are reviewed elsewhere); however, there is also evidence that tolerance might not be a class effect. In contrast to other long-acting nitrates, studies in healthy volunteers showed a preserved vasodilator potency and an absence of oxidative stress and endothelial dysfunction during continuous PETN administration. In animals, PETN was even reported to prevent endothelial dysfunction and atherogenesis in animal models of atherosclerosis. The peculiar properties of this drug, including its capacity to induce the antioxidant defense protein heme oxygenase-1 and to increase the expression and production of ferritin, of the antioxidant molecule bilirubin, and of the vasodilator carbon monoxide have been investigated recently by our group (Figure 8).

Old and New Strategies to Prevent the Development of Tolerance and Cross-Tolerance

Nitrate-Free Interval

Tolerance of the hemodynamic effects of GTN can be avoided by the use of schedules that allow a regenerating daily interval of at least 12 hours. Although this strategy is intrinsically flawed by the fact that patients cannot receive a 24-hour treatment (and typically do not receive nitrate therapy in the early morning hours when the incidence of acute coronary syndromes is highest), it is effective in maintaining the hemodynamic effects of the nitrate. In addition, withdrawal of nitrate therapy results in the development of rebound ischemia, likely associated with the unrestrained effect of endothelial dysfunction and hypersensitivity to vasoconstrictors. The clinical correlate of this phenomenon (reviewed elsewhere) is that, during the nitrate-free interval, the frequency of ischemic episodes is significantly increased, compensating for the benefit of nitrates. In patients with stable angina, Freedman et al have shown an increase in the
duration of silent ischemia compared with patients treated with placebo, and other authors reported a decreased angina threshold after patch removal in both small40 and larger multicenter trials.87 In the catheterization laboratory, acute nitrate withdrawal increased the coronary vasoconstrictor responses to acetylcholine, suggesting that the rebound phenomenon is secondary to an increased sensitivity to vasoconstrictors72; similar findings were reported in animal studies.48,88 Although it is unclear whether the rebound phenomenon occurs only at the vascular level,89 a nitrate-free interval cannot be taken as an acceptable solution.

Interestingly, clinical trials in patients failed to demonstrate the rebound phenomenon in patients with coronary artery disease treated with nitrates and concomitant treatment with ACE inhibitors90 or β-receptor blockers.91 This latter observation emphasizes how exposure to other therapies may alter the pharmacodynamic effects of nitrates and encourages the search for more novel strategies to prevent the development of nitrate side effects.

**Sulphydryl Group Donors**

Following the Needleman and Johnson92 hypothesis that nitrate tolerance could be induced by depletion of the thiol groups necessary for the biotransformation of GTN, a number of animal and human studies have tested the effect of coadministration of sulphydryl donors such as N-acetylcysteine43,93 and L-methionine66,95 with GTN. Almost 20 years later, there is general consensus that these molecules, likely through direct nonenzymatic interaction with GTN, potentiate its effect rather than preventing the development of tolerance. As described above, the identification of critical cysteinyl residues in the active site of ALDH-2 revived this hypothesis, suggesting that agents that “regenerate” the nitrate reductase activity of the ALDH-2 might find a role in the prevention of tolerance. Future research will need to develop clinically applicable thiol donors that may act by the same mechanism such as, for instance, lipoic acid.

**Antioxidants**

A corollary of the oxidative stress hypothesis of nitrate tolerance2 is that treatment with antioxidants may be successful in preventing this phenomenon. Studies published by Bassenge et al96,97 demonstrated that concomitant treatment with a variety of antioxidants preserves the sensitivity of the vasculature to organic nitrates in different animal experimental models. The translation of these results to clinical practice is limited by the fact that oral administration of an effective antioxidant has proved to be more complicated than initially thought.98 This failure appears to mirror the lack of efficacy of oral antioxidants in improving cardiovascular prognosis.99 Although intra-arterial administration of high-dose vitamin C consistently appears to reverse tolerance,66 a positive effect of oral formulations appears to be much less reproducible.98 The development of more potent, more targeted, and more bioavailable antioxidants, particularly targeted at the mitochondria, will address these issues54 (Figure 9).

**Angiotensin-Converting Enzyme Inhibitors and Angiotensin-I Receptor Blockers**

The counterregulatory mechanisms triggered by nitrate therapy may offset the direct vasodilatory effects of GTN and, together with sodium and water retention, may coincide to counterbalance the hemodynamic benefit of these drug. Importantly, the critical role of the renin-angiotensin axis is confirmed by the evidence that ACE inhibitors prevent the development of tolerance in animal100 and human studies.101

In line with the role of ACE inhibitors, nitrate tolerance and nitrate-induced oxidative stress in animal models are markedly attenuated at the level of both conductance and resistance vessels during concomitant administration of angiotensin-I receptor blockers.102 Although it is unclear whether this also applies to humans,103 given the systematic use of ACE inhibitors (eg, in the setting of congestive heart failure), these studies reinforce the concept that nitrate therapy in the modern clinical setting might have totally different implications than what is observed in experimental models (and in former clinical practice).

**Hydralazine**

A favorable interaction between hydralazine and nitrates has been demonstrated in the Veterans Heart Failure Trial (V-HeFT) and in the African-American Heart Failure Trial (A-HeFT).104,105 This particular combination has been shown to have beneficial effects on left ventricular function and exercise capacity; most important, it has been shown to improve survival in large studies in patients with severe heart failure. Although prevention of tolerance is only one of the possible mecha-
isms to explain the benefit associated with this association, hydralazine has also been shown to restore GTN responsiveness in both experimental animals and humans with congestive heart failure. In studies in animal models, the effect of antioxidant carvedilol in preventing tolerance was similar to that of intravenous vitamin C infusions. In studies in patients with arterial hypertension or congestive heart failure, carvedilol, but not atenolol, metoprolol, or doxazosin, preserved the vasodilatory effect of GTN in resistance arteries despite the administration of transdermal GTN in tolerance-inducing doses.

Carvedilol
Third-generation β-blockers such as carvedilol possess additional endotheliotropic properties that might modify the development of tolerance. In animal models, the effect of antioxidant carvedilol in preventing tolerance was similar to that of intravenous vitamin C infusions. In studies in patients with arterial hypertension or congestive heart failure, carvedilol, but not atenolol, metoprolol, or doxazosin, preserved the vasodilatory effect of GTN in resistance arteries despite the administration of transdermal GTN in tolerance-inducing doses.

Statins
Therapy with statins has been associated with a number of benefits that are independent of their effect on lipid levels. In animal studies, both pravastatin and atorvastatin prevented nitrate tolerance and vascular superoxide formation induced by subcutaneous GTN injections, an effect that was associated with increased basal cGMP levels and was abolished when the rats received an inhibitor of the eNOS concomitantly with GTN. These animal data were confirmed by a recent human study showing that administration of atorvastatin prevents both GTN-induced endothelial dysfunction and tolerance in healthy volunteers. Finally, therapy with statins also appears to improve platelet reactivity to GTN in patients with stable or unstable coronary syndromes.

Summary and Clinical Implications
Prolonged exposure to organic nitrates induces tolerance and endothelial dysfunction in patients with cardiovascular disease and healthy volunteers. Although these phenomena have previously been interpreted as the simple loss of a beneficial effect, their prognostic implications remain unexplored, and a recent meta-analysis indicates that long-term therapy with organic nitrates such as ISDN and ISMN may worsen the prognosis of patients with ischemic heart disease.

Complicating the interpretation of these observations, however, clear differences between organic nitrates have been recognized. An interesting example is probably the tetranitrates PETN, which appears to be devoid of tolerance, endothelial dysfunction, and oxidative stress while maintaining the preconditioning-like protective properties. On the basis of current knowledge, organic nitrates can no longer be considered a group of compounds with homogeneous properties and effects but rather a group of substances releasing differing bioactive molecules that lead to distinct implications with respect to tolerance, oxidative stress, and endothelial dysfunction. In line with this, the mechanism of tolerance probably differs significantly across compounds in this class and across vascular beds. Furthermore, we should acknowledge that the implications of GTN-induced stimulation of vascular (mitochondrial) reactive oxygen species are not exclusively negative; a GTN-triggered short-term mitochondrial production of ROS paradoxically plays a role in the preconditioning-mimetic effects of nitrates, which have been proposed to explain the reduced incidence of ST-elevation myocardial infarctions (and improved prognosis) observed in patients undergoing nitrate therapy in the Global Registry of Acute Coronary Events (GRACE) trial. Finally, drugs such as ACE inhibitors, angiotensin-I receptor blockers, l-arginine, BH₄, folic acid, and ascorbate restore the sensitivity to GTN and profoundly modify the vascular effects of nitrates.

In summary, nitrate tolerance (or rather GTN, ISMN, and ISDN tolerance) is a complex phenomenon caused by abnormalities in the biotransformation and signal transduction of nitrates and by activation of counterregulatory mechanisms. Although the issue of the clinical implications of nitrate therapy has not been addressed by randomized clinical trials, it is clear that the pharmacology of organic nitrates is also more complex, interesting, and elusive than previously thought.

Acknowledgments
We greatly appreciate the technical assistance of Margot Neuser.

Sources of Funding
These studies were supported in part by a grant from the Federal Ministry of Education and Research (BMBF 01EO1003), by the Deutsche Forschungsgemeinschaft, and by vascular biology grants from ACTAVIS.

Disclosures
Drs Münzel and Gori have received honoraria from ACTAVIS for lectures. Dr Daiber has received honoraria and a vascular biology grant from ACTAVIS.

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