NITRATE TOLERANCE
A UNIFYING HYPOTHESIS

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The first part of this review provided a synopsis of the recent literature about superoxide anion (O2−) production, endo-
theelial dysfunction, and the neurohormonal activation that follow long-term administration of organic nitrates. In this issue of Circulation, we will try to integrate these observations with other separate, and, to a certain extent, antagonistic hypotheses that have been proposed for the development of nitrate tolerance.1–3 Hypotheses concerning the pathogenesis of tolerance have traditionally been grouped into 2 different categories. The “dispositional” or “metabolic” theory postulates that the effect of organic nitrates wanes during continuous use as the result of decreased biotransformation or decreased activity of the nitric oxide (NO) adjunct released in this process (end-organ tolerance). The “functional” theory emphasizes the importance of counterregulatory mechanisms that occur in response to nitrate therapy, including neurohormonal activation and plasma volume expansion. These mechanisms could counterbalance and overcome the effects of nitrates, a process that has been termed "pseudotolerance."3,4

Recent findings described in the first part of this article provide an opportunity to hypothesize explanations for a number of previous observations in the field of nitrate tolerance by applying increased O2− production as the underlying mechanism. In the following text, the evidence for this concept is reviewed, and a unifying hypothesis based on a self-promoting mechanism triggered by increased vascular O2− generation is proposed.

Plasma Volume Expansion During Nitrate Therapy

Several studies reported that nitrate therapy causes plasma volume expansion, as demonstrated by a decreased hematocrit during nitrate therapy both in healthy volunteers and patients with congestive heart failure or ischemic heart disease. This observation led to the hypothesis that increased circulating volume and, subsequently, filling pressures, would counteract the nitroglycerin (GTN)–induced decrease in preload, thus causing nitrate tolerance. Increased water retention and/or fluid shifts from the extravascular to the intravascular compartment have been proposed as mechanisms for these changes.5–7 The activation of the renin-angiotensin-aldosterone axis and the increased angiotensin II production during tolerance might mediate both processes. Furthermore, the recent demonstration that GTN treatment causes increased levels of isoprostanes,8 given their direct vasoconstrictor and antinatriuretic effects, provides an additional, redox-mediated explanation. Finally, changes in the redox state in the endothelial cellular milieu, and, in particular, changes in the availability of reduced thiol groups, might induce abnormalities in microvascular permeability.10

Despite these theories, a number of observations limit the importance of plasma volume expansion as a causal mechanism of tolerance, including: (1) the discrepancy in the time course of the two phenomena; (2) the demonstration that tolerance can be induced in isolated vessels; and (3) the lack of preventive effect of diuretics.11,12 These observations suggest, however, that plasma volume expansion might be one of the effects induced, at least in part, by an increased oxygen free radical production.

Abnormalities in Organic Nitrate Biotransformation

Abnormalities in the biotransformation process through which the nitrate undergoes denitrification to yield NO or some NO adjunct(s) were proposed as a mechanism of tolerance almost 3 decades ago.13 Despite the existence of negative reports,14 new interest in this hypothesis has developed. The concept that nitrate biotransformation becomes impaired finds support in a number of studies demonstrating that tolerance is accompanied by reduced formation of the GTN metabolite 1,2 dinitrate.15,16 An impaired biotransformation of GTN and other nitrates, such as isosorbide mononitrate and dinitrate, would also help explain the finding that spontaneous NO donors, such as nitroprusside and other nonorganic NO donors, are subject to relatively lesser degrees of tolerance.17–20

Whether reduced biotransformation is a relevant mechanism of tolerance, and, most importantly, which enzymes are involved in the activation of GTN, will require further investigation. The microsomal cytochrome P450, glutathione transferases, NAD(P)H and xanthine oxidases, and, finally,
given its cytochrome-like structure, nitric oxide synthase (NOS), have all been proposed. However, incubation with inhibitors of these enzymes blunted GTN responses to the same extent in tolerant and nontolerant vessels, demonstrating that tolerance is not associated with any decrease in their activity. To date, GTN biotransformation has been demonstrated to be inhibited by NO, but its redox sensitivity has not been formally investigated.

Decreased bioavailability of reduced thiols necessary for GTN biotransformation was originally identified by Needleman et al. as the cause of tolerance. It now seems that free thiol groups are not a necessary substrate for NO release from GTN and that they are not depleted during nitrate exposure. However, oxidation of protein-bound thiols resulting from an alteration in cellular -O2·- bioavailability might compromise the function of different enzymes, including NOS and membrane enzymes, resulting in a free radical–mediated inhibition of GTN biotransformation in the setting of tolerance. Furthermore, multiple studies have demonstrated that changes in redox state or increased peroxynitrite production can impair the activity of heme proteins, such as cytochrome P450 and NOS, and can also inhibit glutathione S-transferases, another family of enzymes potentially involved in GTN biotransformation. Finally, Chen et al. provided evidence that the mitochondrial aldehyde reductase is able to catalyze the production of nitrite and 1,2 glyceryl dinitrate through a process that seems to depend on reduced sulphydryl groups. The subcellular location of this enzyme might make it particularly susceptible to oxidative changes, as high mitochondrial concentrations of NO inhibit the respiratory chain, thus inducing ·O2·- generation. In sum, impaired GTN transformation might participate in the development of tolerance, although it cannot explain many of the abnormalities observed in this setting. Future research will have to formally address the ·O2·-sensitivity of GTN biotransformation.

Abnormalities in NO Signal Transduction

A number of studies have suggested that prolonged exposure to nitrates might diminish not only the bioavailability but also the efficacy of their active metabolite NO. The GTN-released NO activates the enzyme-soluble guanylyl cyclase (sGC) in smooth muscle cells, thus increasing tissue levels of the second messenger cGMP, which, in turn, leads to the activation of a cGMP-dependent protein kinase (cGK) (Figure 1). This enzyme mediates vasorelaxation through the phosphorylation of different proteins involved in the regulation of intracellular Ca2+ levels. End-organ tolerance might be mediated by decreased activity of the sGC, increased activity of phosphodiesterases (PDE, the enzymes responsible for cellular cGMP catabolism), or decreased activity of the cGK, the final effector of the system. The former 2 mechanisms would reduce the bioavailability of the intracellular second messenger cGMP, whereas the latter would impair its effects. Modifications in the activity of these enzymes might explain the partial decrease in the responses to endothelium-dependent and other NO-dependent vasodilators.

Augmented catabolism by PDEs has been proposed as a mechanism that leads to reduced bioavailability of cGMP in the setting of tolerance, and a recent report demonstrated that the activity of the PDE 1A1 is increased in rats treated with continuous GTN. This finding, as the authors suggest, is consistent with both reduced responses to NO-dependent vasodilators and increased responses to vasoconstrictors that augment intracellular Ca2+ concentrations, such as angiotensin II and norepinephrine (Figure 1).

The effect of GTN on NO signaling mechanisms and the possibility that a dysfunction might be caused by increased free radical production were also recently investigated. Despite an increased basal expression of its components, the activity of the sGC-cGK pathway was significantly blunted, as assessed by the assay of the vasodilator-stimulated phosphoprotein, a marker of cGK activity. Both in vitro and in vivo treatment with vitamin C prevented the dysfunction of these enzymatic pathways, confirming the role of increased ·O2·- bioavailability in the development of end-organ tolerance. Different studies have now demonstrated that elevated concentrations of peroxynitrite and ·O2·- alone inhibit sGC, possibly through oxidation of thiol groups in its catalytic site that seem to be critical for the direct activation of the enzyme by GTN. Finally, in the presence of an excess of ·O2·- and/or peroxynitrite, the activity of ion channels involved in the regulation of Ca2+ and K+ currents (which are the final mediators of GTN-induced vasodilatation) seems to be compromised. Taken together, these lines of evidence suggest the existence of a fundamental mechanism, based on increased ·O2·- generation, that leads to several of the abnormalities observed in nitrate tolerance.

![Figure 1. Mechanisms of action of GTN. GTN is metabolized to NO, which stimulates the synthesis of cGMP. In turn, cGMP reduces cytoplasmic Ca2+ by inhibiting inflow and stimulating mitochondrial uptake, causing relaxation of smooth muscle cells. The role of Ca2+-activated K+ currents is also being investigated; by inducing hyperpolarization of the cellular membrane, they might contribute to the limitation of Ca2+ entry in smooth muscle cells, and, in endothelial cells, they might inhibit ·O2·- production. By promoting Ca2+ uptake, ATII can increase cytoplasmic Ca2+ and induce the Ca2+-dependent PDE1A1. This may lead to reduced cGMP and cGK activity, providing an elegant explanation for both nitrate tolerance and increased sensitivity to ATII. There is evidence supporting a redox sensitivity of all enzymes involved in these processes.](Image)
The Trigger Mechanism

Therapy with organic nitrates is associated with a complex series of responses that seem to mediate the phenomenon of tolerance in a multifactorial fashion. At the moment, the most convincing hypothesis suggests that therapy with organic nitrates, particularly GTN, is associated with increased $\cdot O_2^-\,$ bioavailability. As was discussed, there seem to be multiple potential sources for this $\cdot O_2^-\,$. Importantly, the initial step in this mechanistic cascade remains to be determined, and at this time investigative efforts need to realize the primary trigger of this increase in free radical production. Increased $\cdot O_2^-\,$ bioavailability might explain a number of the abnormalities described during nitrate therapy, including direct GTN-derived NO quenching, NOS uncoupling (and thus further reduced NO and increased $O_2$ generation), increased production of potentially harmful mediators such as peroxynitrite and isoprostanes, increased sympathetic activity, and possibly also decreased end-organ effect and biotransformation of GTN. In light of this, a determination of the original trigger of this abnormal production remains a critical component of our understanding of these events.

Importantly, recent studies have demonstrated that $\cdot O_2^-\,$ seems to be generated instantaneously on bolus GTN administration, suggesting that it might be a direct by-product of GTN biotransformation. It is possible that this initial increase in $\cdot O_2^-\,$ could play a role as primary stimulus for a series of events that eventually lead to the development of both tolerance and endothelial dysfunction. Indeed, if $\cdot O_2^-\,$ is released directly after GTN administration, increased peroxynitrite formation could result from the interaction between $\cdot O_2^-\,$ and the NO released from GTN. This generation of $O_2$ and peroxynitrite, intrinsic in the administration of GTN, might provoke a series of autocatalytic mechanisms leading to further redox unbalance (Figure 2) and, ultimately, tolerance.

Perspectives

In conclusion, despite the fact that there continue to be many uncertainties, recent findings allow the formulation of a "unifying" hypothesis of nitrate tolerance that has its fundamental basis in an increased $O_2$ production. More research is now necessary to further substantiate the redox sensitivity of GTN biotransformation, NO signal transduction, volume expansion, and other recently described pathways (e.g., endothelial Ca$^{2+}$-activated K$^+$ channels).

From the clinical point of view, many strategies have been used in an effort to prevent or modify nitrate tolerance. The failure of these strategies to produce a definitive solution has often been attributed to the multifactorial nature of this phenomenon. We believe that recent insights into the mechanism of tolerance have 2 important implications. First, the concept that many systemic and local abnormalities induced by GTN treatment might arise from a common origin would suggest that, once the original source of $O_2$ is identified, a rational, targeted approach to the prevention of tolerance and nitrate-induced endothelial dysfunction could be developed. Second, the demonstration that organic nitrates can cause free radical production, endothelial dysfunction, and sympathetic activation would suggest that nitrate therapy may have long-term detrimental effects. Large-scale prospective studies of the use of nitrates in coronary artery disease were relatively short and produced controversial results. At the moment, one can only conclude that we have no hard data concerning the impact of long-term nitrate therapy on clinical outcome.

GTN seems to increase matrix metalloproteinases activity (possibly rising the risk of plaque rupture), and a recent meta-analysis of patients after a myocardial infarction showed increased risk of cardiac death in patients treated with organic nitrates. Therefore, in the absence of more definitive data, the knowledge that therapy with organic nitrates causes both increased $O_2$ production and endothelial dysfunction places a cautionary note on the traditional assumptions about their beneficial effects.

Acknowledgments

Dr Gori is the recipient of a research fellowship from the Heart and Stroke Foundation of Ontario, Canada. Dr Parker is a Career Investigator of the same institution.

References


Figure 2. Diagram describing the different mechanisms through which an initial increased production of oxidant free radicals might trigger a series of autocatalytic processes leading to further $O_2$ and peroxynitrite generation. Superoxide anion and peroxynitrite may result in NOS uncoupling, sympathetic activation, SOD inhibition, and increased production and responsiveness to vasoconstrictors such as angiotensin II. These phenomena, in turn, might lead to further increases in oxidative stress. SOD indicates superoxide dismutase; ATII, angiotensin II; and ET-1, endothelin-1.

Key Words: acetylcholine | nitroglycerin | endothelium | nitric oxide synthase | blood flow
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Circulation. 2002;106:2510-2513
doi: 10.1161/01.CIR.0000036743.07406.53
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2002 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/106/19/2510

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