Mini-Review

The Puzzle of Nitrate Tolerance
Pieces Smaller Than We Thought?

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Nitroglycerin (GTN) and other organic nitrates are important drugs commonly used in cardiovascular medicine, and, more recently, in obstetrics as tocolytic agents. The development of tolerance, ie, the reduction in effect or the requirement for higher doses that appears after continuous use, is a major factor limiting the efficacy of these drugs. Despite their clinical importance in the therapy of ischemic heart disease and heart failure, many aspects of the pharmacology of organic nitrates, including the mechanism(s) of tolerance, remain unclear.

In the past decade, studies have demonstrated that organic nitrate therapy leads to complex interactions between the vasculature, neurohormones, and free oxygen radicals. In particular, the concept that GTN treatment causes increased vascular superoxide anion ($\cdot$O$_2^-$) production, the mechanisms leading to this production, and the consequences of this phenomenon on endothelial function, have all been investigated. In the first part of this 2-part review, these recent findings, as well as the potential role of neurohormonal and autonomic abnormalities, will be described. In the second part, which will appear in the next issue of Circulation, we will propose a new, integrated view on the pathophysiology of nitrate tolerance.

The Vascular Free Radical Hypothesis of Nitrate Tolerance

There is evidence from both animal and human studies that nitrate tolerance, especially when induced in vivo, is associated with an increased bioavailability of $\cdot$O$_2^-$, and that this process is responsible for the development of tolerance. Superoxide anion is normally scavenged by multiple intracellular and extracellular mechanisms, including the enzyme superoxide dismutase (SOD). However, in higher concentrations, it can overcome these mechanisms and rapidly react with nitric oxide (NO), the active metabolite of GTN, to form peroxynitrite. This may reduce the bioavailability of GTN-derived NO, impairing its vasodilator activity (and that of other NO donors), and, possibly, directly counteract GTN-induced vasorelaxation, because even in small concentrations, peroxynitrite has a direct vasoconstrictor effect.

Signs of increased $\cdot$O$_2^-$ and peroxynitrite production during continuous nitrate therapy have been demonstrated in both animals and humans. Therefore, an $\cdot$O$_2^-$-mediated mechanism has been proposed as the cause of tolerance (Figure 1).

Experiments involving the concurrent administration of GTN and antioxidants appear to support this free radical hypothesis. In vitro, coincubation of vascular tissue with liposome-encapsulated SOD can partially restore GTN responses, and the inhibition of this enzyme mimics tolerance. In vivo, supplementation with the antioxidant vitamin C prevented tolerance both in animal and in human studies, despite being unable to reverse it.

In sum, there is now a body of evidence suggesting that some nitrites, particularly GTN, are associated with increased vascular oxygen free radical generation, and that these responses are involved in the genesis of tolerance. The sources of $\cdot$O$_2^-$ that have been proposed include membrane NAD(P)H oxidases in the response to therapy with GTN. The importance of NAD(P)H oxidases in the response to therapy with GTN has been demonstrated in a number of animal studies, and an important regulatory involvement of angiotensin II has been proposed. Interestingly, the importance of these enzymes has been demonstrated in studies in which hydralazine, by inhibiting NAD(P)H oxidases, modified the development of tolerance in both animals and humans. Although these membrane-bound oxidases are located in both smooth muscle and endothelial cells, the preponderance of evidence points to a crucial role of the endothelium in $\cdot$O$_2^-$ production, suggesting that the culprit enzyme(s) are within this tissue.

GTN and the Endothelium

Clear evidence now exists that GTN therapy has negative effects on the function of the NOS, the enzyme responsible for the endothelial control of vascular tone. Evidence of this effect has now been developed in multiple animal and human models, including the coronary circulation of patients with ischemic heart disease. In particular, in vitro experiments have reported increased expression but decreased activity of NOS during continuous GTN, associated with increased $\cdot$O$_2^-$ generation. GTN therapy appears to induce a dysfunctional state of NOS, in which the reductive activation of molecular...
oxygen to form $\cdot O_2^-$ is not followed by oxidation of L-arginine and NO synthesis, resulting in the net generation of $\cdot O_2^-$—a situation termed “NOS uncoupling.” Uncoupling can be triggered by reduced bioavailability of tetrahydrobiopterin and/or L-arginine, respectively a cofactor and the substrate for NOS (Figure 2).23,24

Animal studies demonstrated that peroxynitrite is able to oxidize tetrahydrobiopterin to the ineffective form dihydrobiopterin,25 thus providing a potential mechanism in GTN tolerance. In this positive feedback mechanism, a peroxynitrite-dependent oxidation of tetrahydrobiopterin would cause NOS to produce even greater quantities of $\cdot O_2^-$. Evidence for this proposed mechanism can be found in a report by Gruhn et al26 in which the administration of tetrahydrobiopterin was found to restore the activity of NOS. Despite this effect, in this animal model, supplemental tetrahydrobiopterin did not reverse tolerance.

A number of studies have suggested that folic acid supplementation can improve endothelium-dependent vascular function in the presence of multiple risk factors for cardiovascular diseases.27 Our laboratory recently tested, in a group of healthy volunteers, the hypothesis that this vitamin would have a favorable impact on the abnormalities induced by nitrate therapy at the level of the endothelium. We documented that supplemental folic acid is able to prevent the development of both NOS dysfunction and nitrate tolerance as assessed by forearm plethysmographic measures of arterial blood flow.28 The mechanism of this effect remains unknown, but a number of possibilities can be proposed, including: (1) a folate-mediated increase in tetrahydrobiopterin regeneration with subsequent NOS recoupling;29,30 (2) inhibition of the other sources of $\cdot O_2^-$, such as xanthine and/or NADPH membrane oxidases;29,30 (3) depletion of NAD(P)H reserves;31 (4) direct antioxidant effect;32 and, finally (5) direct substitution of tetrahydrobiopterin as a cofactor for NOS.33 Whatever the mechanism, it appears that, during GTN treatment, NOS uncoupling might be both cause and effect (via tetrahydrobiopterin oxidation) of the described increase in vascular $\cdot O_2^-$ generation.

Another hypothesis was recently developed with regard to the mechanism of GTN-induced NOS uncoupling with subsequent increases in $\cdot O_2^-$ bioavailability. This hypothesis states that GTN therapy leads to intracellular L-arginine depletion. Although the exact mechanism remains controversial, it has been demonstrated that NO donors, including GTN, activate NOS.34 The subsequent increase in L-arginine demand and the negative effect of sustained administration of NO donors on L-arginine uptake35,36 may lead to L-arginine depletion, resulting in the uncoupling of NOS and increased $\cdot O_2^-$ generation. It is now recognized that the arginine transporter and NOS are colocalized within caveolae of the endothelial cell, and that changes in arginine concentration may not be uniform throughout the endothelial cell.37 These observations have led to the arginine depletion hypothesis of nitrate tolerance. In this case, therapy with nitrates leads to intracellular L-arginine depletion with resultant uncoupling of NOS and increased $\cdot O_2^-$ production. Of note, there is experimental evidence to suggest that L-arginine has antioxidant effects as a scavenger for $\cdot O_2^-$,38 a characteristic that may also play a role in modifying responses to GTN therapy. In vitro studies have demonstrated that L-arginine supplementation prevents the uncoupling of NOS during nitrate exposure and modifies the development of tolerance.34 Finally, a recent study39 in patients with angina documented that supplemental L-arginine prevents the development of tolerance during sustained treatment with continuous transdermal GTN.

**Neurohormonal Activation and the Control of $\cdot O_2^-$ Production**

The neurohormonal activation hypothesis of nitrate tolerance was developed from the evidence that GTN stimulates counterregulatory responses mediated by sympathetic nervous system and renin-angiotensin-aldosterone axis.40–43 Although the vasoconstriction induced by these responses has been implicated in the rebound ischemia after GTN withdrawal,44–47 a direct role in the development of tolerance has never been substantiated.41,48

Figure 1. Diagram describing the oxidative pathway for the development of nitrate tolerance. Both endothelial and smooth muscle cells are involved in these processes. Increased free radical generation, increased production and responsiveness to angiotensin II, and L-arginine depletion may result in NOS uncoupling and further production of $\cdot O_2^-$ and peroxynitrite. ET-1 indicates endothelin-1; BH4, tetrahydrobiopterin; AT II, angiotensin II; and $\cdot O_2^-$, superoxide anion.
O₂⁻ inhibition of NOS, and a recent report demonstrated that L-arginine supplementation can reverse this process. NOS equilibrium of BH₄. During nitrate tolerance, this equilibrium might be shifted toward the uncoupled form of the enzyme. In turn, O₂⁻ and peroxynitrite can oxidize BH₄, resulting in further uncoupling. Folic acid (possibly though a BH₄-mediated mechanism) and L-arginine supplementation can reverse this process. NOS III indicates endothelial nitric oxide synthase; BH₄, tetrahydrobiopterin; and BH₂, dihydrobiopterin.

Interestingly, recent evidence suggests that neurohormonal responses may play a role in free radical generation. Angiotensin II production appears to play a permissive or causal role in nitrate-induced increased O₂⁻ via angiotensin II–stimulated membrane NAD(P)H oxidases. In humans, angiotensin II showed a direct negative effect on vascular responsiveness to GTN. In animal studies, the administration of ACE inhibitors or an angiotensin II receptor blocker, reduced O₂⁻ production and prevented GTN tolerance, thus reinforcing the idea of a pro-oxidant effect of this mediator.

There is also evidence for a role of endothelin in the development of tolerance. The production of endothelin is stimulated by both angiotensin II and oxidative stress in endothelial and smooth muscle cells. An increased endothelin-1–like and big endothelin-1–like immunoreactivity in the tunica media of tolerant vessels has been demonstrated during nitrate treatment in two different animal models. In one report, exposure of normal vessels to low concentrations of endothelin-1 caused a hypersensitivity to vasoconstricting agents similar to that observed in nitrate tolerant vessels. In rabbits, an endothelin-1 receptor inhibitor reduced O₂⁻ production and the development of GTN tolerance, although angiotensin II receptor inhibitors appeared more effective. The effects of endothelin-1 include direct vasoconstriction, a positive feedback increase of angiotensin II production, and the activation of protein kinase C (PKC). In turn, PKC stimulates a NAD(P)H oxidase-mediated O₂⁻ production and might be autokalytically activated by O₂⁻. In addition, PKC-mediated phosphorylation causes inhibition of NOS, and a recent report demonstrated that angiotensin II, via an O₂⁻ and PKC-mediated mechanism, can trigger NOS uncoupling. Inhibition of PKC prevented tolerance in a rat model, but the effect of endothelin-1 receptor or PKC inhibition in the response to nitrates in humans has not yet been tested.

One of the major controversies about the traditional neurohormonal hypothesis is that tolerance can be induced in isolated vessels, thus excluding the importance of circulating plasma levels of any mediator. The recognition that neurohormonal mediators may be produced by vascular cells and may induce tolerance by increasing local O₂⁻ generation, i.e., via paracrine or autocrine pathways, suggests that this hypothesis is not inconsistent with such observations. Local coronary and/or systemic abnormalities in the endothelial neurohormonal milieu could play a role in tolerance, may lead to abnormalities in NOS function and, finally, could also be involved in the rebound phenomena that follow abrupt cessation of GTN therapy.

### Autonomic Nervous System Responses to Nitrate Therapy

Observed increases in (nor)epinephrine, endothelin-1, and angiotensin II levels after nitrate treatment might be mediated in part by the sympathetic nervous system. The role of autonomic responses in the development of tolerance has been the subject of a number of studies and considerable controversy. Experimental evidence suggests that GTN does interact with the autonomic nervous system and that this interaction is complex and occurs at peripheral and central sites. In particular, NO synthesis in the brain stem appears to have chronic inhibitory effects on medullary areas modulating sympathetic outflow. This effect seems to be lost in nitrate tolerance, possibly as a result of NOS dysfunction in the central nervous system. Increases in central nervous system O₂⁻ and angiotensin II production are felt to play a role in mediating these heightened sympathetic responses during sustained nitrate therapy. This tolerance-dependent loss of a sympathoinhibitory mechanism, termed “sympathetic tolerance,” might blunt the vasodilatory responses to NO donors in resistance vessels, thus contributing to GTN tolerance. Therefore, nitrates may influence the autonomic nervous system at several locations. Although the net effect of these responses represents a complex function that is also dependent on whether the nitrate exposure is acute or chronic, sustained therapy with organic nitrates appears to lead to a relative increase in sympathetic outflow associated with a parallel decrease in parasympathetic tone.

In conclusion, recent evidence suggests that abnormalities in autonomic nervous system regulation, renin release, and redox state are strongly integrated and form a complex mechanistic puzzle, the fundamental pieces of which appear to be small and elusive oxygen-free radicals. The findings described above are important not only because of their potential role in the mechanism of tolerance, but also because neurohormonal and autonomic activation, O₂⁻ generation, and NOS dysfunction might have prognostic relevance in heart failure and coronary artery disease. In the next issue of *Circulation*, we will propose the existence of mechanistic links between these phenomena and other abnormalities that are present during tolerance and provide our perspective view.
on research and clinical issues related to the use of organic nitrates.

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References

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