Discovered and patented as an explosive by Alfred Nobel in the 1860s, nitroglycerin has been formulated for use in the treatment of symptomatic CAD for over 140 years. In fact, later in life, Nobel himself was prescribed the medication for angina, but refused to take it because of the associated side effect of headache.

With glycerol trinitrate (GTN) as the prototype, nitrates represent one of the safest and most rapidly effective pharmacological means to reduce acute symptoms of myocardial ischemia attributable to obstructive coronary disease. This has led, over the years, to the development of long-acting oral and topical preparations. However, efficacy with chronic administration is more difficult to achieve because of the development of therapeutic resistance, generally occurring a few days after initiating treatment. This phenomenon known as nitrate tolerance has been the stimulus for intense investigation of the metabolic fate of nitroglycerin with the idea that modulation of its biotransformation could improve efficacy of chronic treatment.

The mechanism of GTN-induced dilation is complex and was not identified until more than 100 years after its discovery. GTN is not a direct vasodilator, rather it must be converted to dinitrate products for vasoactivity. Biotransformation to the active metabolite nitric oxide (NO) occurs in parallel with the formation of glycerol-1,2-dinitrate and involves a dithiol-dependent process.

It was not until recently that the principal enzyme responsible for biotransformation of GTN was identified. Chen et al. showed that mitochondrial aldehyde dehydrogenase (ALDH-2) metabolizes GTN to glycerol-1,2-dinitrate and nitrite. This was confirmed by Sydow et al. using mitochondrial-deficient cultured endothelial cells, although a cytosolic source of ALDH-2 has also been suggested. The mitochondrial enzyme converts nanomolar concentrations of GTN to active nitrodrilator metabolites in vivo and in vitro, as shown by direct measurements coupled with the use of selective inhibitors and competing substrates.

Whether the metabolic product NO or a related compound is responsible for the subsequent activation of guanylate cyclase remains in question. Other enzymatic pathways of GTN metabolism include glutathione-S-transferase and cytochrome P450 reductase, but neither leads to the formation of glycerol-1,2-dinitrate. Glutathione-S-transferase metabolizes GTN to the inactive product glycerol-1,3-dinitrite and is thought to represent a pathway for metabolic inactivation of GTN. Cytochrome P450 enzymes are capable of active biotransformation of nitrates, but the Km for the enzyme is such that generation of NO occurs only at high (micromolar) GTN concentrations.

The differential vascular distribution of cytochrome P450 enzymes may explain spatial regional variability in nitrodrilator effects as well as the greater response in venous than arterial segments. Which of these metabolic pathways are activated depends on both the concentration of the nitrate compound used as well as the number of nitrate groups present on the parent compound. Thus ISDN and ISMN are not bioactivated by ALDH-2, but GTN and PETN are.

The cell type responsible for nitroglycerin biotransformation is not well-defined because the enzymes involved are expressed throughout the vascular wall. Some evidence indicates that vascular smooth muscle is responsible for conversion to active metabolites. However, recent data suggest that the endothelium is important because dilation to GTN is reduced substantially by endothelial removal.

Nitrate tolerance was demonstrated more than 100 years ago, but the mechanism appears complex and is only now being unraveled (nicely reviewed in13,14). There are 2 processes involved which result in tolerance to chronic nitrate therapy, cross-tolerance to other NO donors, and endothelial dysfunction. First, chronic nitrate therapy increases vascular oxidative stress and reduces bioavailability of NO. The resulting reactive oxygen and nitrogen species inhibit bioactivation of the administered nitrate.

Tolerance occurs via GTN-induced production of superoxide, likely from mitochondrial sources because mitochondrial-targeted antioxidants prevent it and heterozygous MnSOD-deficient mice show heightened sensitivity. However, other sources exist including NADPH oxidase and uncoupled NOS, either through BH4 oxidation or depletion of intracellular arginine. The culprit superoxide is derived both from vascular smooth muscle and endothelium, as denudation reduces both tolerance to NTG and cross tolerance to other NO donors. An increase in PKC activity also occurs with nitrate tolerance, contributing to enhanced constriction, reduced dilation, and NOS uncoupling.

Second, in addition to quenching NO that is derived from GTN, ROS, including NO, can block bioactivation of GTN by disulfide modification and inhibition of ALDH-2. Thus GTN induces tolerance both by reducing GTN biotransformation to dilator metabolites (inhibition of ALDH-2) and...
by increasing mitochondrially produced ROS which inactivate those metabolites, specifically NO, yielding additional radical species. Multiple feedback inhibitory systems exist whereby superoxide and the byproduct of NO and superoxide, peroxynitrite, inhibit ALDH-2, resulting in a tolerant state (see the Figure). Cytochrome P450 enzymes involved in nitrate bioactivation are also downregulated in response to a 48-hour infusion of nitroglycerin, whereas induction of these enzymes prevents tolerance in a rat model.22

Understanding the mechanism of tolerance may suggest interventions to reduce it. For example, BH4 supplementation in rats treated with NTG reverses the associated endothelial dysfunction, presumably by reducing superoxide formed from uncoupled NOS3.23 Folic acid, necessary for BH4 synthesis, attenuates nitrate tolerance in the human forearm.24 Both through quenching NO and possibly stimulating additional production of intracellular ROS (ROS-induced ROS release25), GTN-induced mitochondrial-derived ROS effectively reduce the amount of bioavailable NO and induce tolerance to other NO donors. The reduced bioavailability of NO is also manifest as endothelial dysfunction with reduced dilation to agonists that operate through the endothelial generation of NO.

Biochemical pathways mediating nitrate bioactivation (black) and tolerance (red). Stimulatory pathways are designated by solid lines with arrows. Inhibitory pathways are denoted by dashed arrows with diamond heads. Glycerol trinitrate (GTN) and pentaerythritol tetranitrate (PETN) are activated by the mitochondrial isoform of aldehyde dehydrogenase (ALDH-2) to NO and the corresponding dinitrates, 1,2-GDN and PEDN, respectively. Larger concentrations of PETN (and isosorbide dinitrate; not shown) yield NO through activity of cytochrome P450 reductase. Glutathione-S-transferase inactivates GTN to 1,3-GDN. Nitrates also stimulate superoxide production from mitochondria and other sources (uncoupled NOS3, NADPH oxidase), quenching NO and producing peroxynitrite. These redox active products inhibit ALDH2 activity by oxidizing critical cysteine residues, blocking biotransformation of nitrates and producing tolerance. Unlike GTN, PETN activates heme oxygenase-1, augmenting antioxidant production of bilirubin and ferritin. This prevents the rise in oxidative potential, maintains NO bioavailability, and prevents the tolerant state. Hemin, statins, the apo-A1 mimetic D-4F also stimulate HO-1, whereas apigenin blocks HO-1, resulting in tolerance to PETN. A variety of antioxidants can also avert nitrate tolerance by reducing superoxide. ANP indicates atrial natriuretic peptide; CO, carbon monoxide; BH4, tetrahydrobiopterin.

The study by Wenzel et al26 in this issue of Arteriosclerosis, Thrombosis, and Vascular Biology importantly extends observations made in vitro regarding the mechanism of nitrate tolerance to the in vivo setting. It also sheds new light on the mechanism of ROS generation during chronic nitrate therapy by examining the difference between the effects of GTN and PETN, the latter being a nitrate compound that does not elicit tolerance.26 Interestingly both nitrate formulations must undergo metabolic activation via the same mitochondrial enzymatic pathway (ALDH-2; Figure). However Wenzel et al found that chronic treatment with PETN but not NTG elevated heme oxygenase (HO-1) expression and activity.26 The byproduct of HO-1 activity, bilirubin, and the associated upregulation of ferritin by PETN are potent intracellular antioxidants. Upregulation of HO-1 and bilirubin levels by PTEN treatment has been demonstrated previously in endothelial cell culture.27

To confirm the role of HO-1 in nitrate tolerance, HO-1 was stimulated using hemin. In this situation GTN treatment no longer generated ROS or resulted in tolerance. Conversely, when animals were treated with an inhibitor of HO-1, apigenin, PETN no longer increased levels of bilirubin or
ferritin, and tolerance was observed.26 These findings strongly suggest that endogenous HO-1 modulates nitrate-induced ROS generation, eliminating the paradoxical quenching of NO and thereby preventing tolerance. This observation is corroborated by indirect evidence for enhanced cGMP-dependent protein kinase-I activation in aortas of PETN-treated but not NTG-treated animals.

Wenzel et al26 make a compelling case for endogenous HO-1 in preventing tolerance to PETN, but a few questions remain. The approach used is largely pharmacological with attendant problems of nonspecificity. Use of HO-1 knock-out mice28 and wild-type controls would help obviate this concern. It is unclear whether the antioxidant effect of HO-1 involves the production of bilirubin, increased expression of ferritin, or both actions of this enzyme.29 Future studies should help resolve this issue and better define the role of nonmitochondrial sources of ROS that might be involved in the initiation or maintenance of tolerance.

The study by Wenzel sheds light on an important endogenous mechanism, HO-1, the activation of which can avert nitrate tolerance. Other means to upregulate HO-1 such as piceatannol, a phytochemical,30 or naturelupeires (ANP)31 might also prevent tolerance when combined with chronically administered nitrate preparations. A novel apoA1 mimetic, D-4F, which has antioxidant properties, also upregulates HO-1.32 Finally, statins stimulate HO-1 activity33 and protect against nitrate-induced oxidative stress in eNOS KO mice.34 Indeed statins may be useful adjunct therapy in the prevention of nitrate tolerance.35

The clinical and physiological ramifications of this study are significant. By shifting the cellular redox state toward an excess of reactive oxygen and nitrogen species, traditional nitrate medications elicit endothelial dysfunction and activation of vascular smooth muscle cells, generating a cascade of detrimental effects similar to those imposed by CV risk factors. This is supported by the present study where both GTN and PETN initially reduced arterial pressure but by 6 days of therapy a frank increase in pressure was observed with GTN treatment, whereas the lower pressure persisted in the PETN group.26 This suggests that the excess production of ROS quenches not only the NO produced by bioactivation, but also endogenously released and important signaling concentrations of NO. Thus with some nitrate formulations, the early symptomatic improvement of angina may give way to a potentially harmful response with chronic administration. Indeed an analysis of most36 (but not all37) clinical data suggests this is the case. If borne out by future studies, this concern could explain why nitrates have not been associated with a mortality benefit in coronary disease, unlike other treatments for cardiovascular disease such as aspirin, beta blockers, and angiotensin converting enzyme inhibitors. It also explains why combined treatment with antioxidants (eg, vitamin C38, hydralazine,16 Tempol39) can reduce the ROS generation and tolerance that occur with chronic nitrate use. An alternative strategy is suggested by the current article involving PETN, a nitrate dilator that concomitantly activates endogenous antioxidant mechanisms and avoids tolerance.

Vascular risk factors, through the associated reduction in NO bioavailability, create a milieu for the development and progression of atherosclerosis. It has been suggested that nitrate therapy, through the generation of NO, might suppress this vascular proliferative transformation.19 However, chronic treatment with traditional nitrate compounds would be ineffective because of the resulting elevation in ROS. A compound such as PTEN that is devoid of excessive oxidant generation might have promise. Indeed Kojda et al40,41 showed that compared with ISMN, PTEN prevented endothelial dysfunction and reduced the development of atherosclerosis in the aorta of cholesterol treated New Zealand White rabbits. Additional preclinical studies are warranted to pursue this observation.

In summary, our understanding of nitrate bioactivation and induction of tolerance has advanced significantly in the past decade. Metabolic activation of exogenous nitrate compounds requires mitochondrial ALDH-2 and cytochrome P450 enzyme systems resulting in the production of NO and other nitrodilators. Enzymatic activation also augments the oxidative environment resulting in feedback inhibition of the biotransformation and quenching of the generated NO. This mechanism of tolerance to nitrate therapy can be reduced by concomitant upregulation of endogenous HO-1 which occurs with PETN, but not with administration of typical clinically used nitrate compounds. Future research should focus on novel non–oxidant-generating nitrate formulations or adjunctive therapy with targeted antioxidant compounds (eg, mitochondrial targeted antioxidants42,43) to obviate the associated tolerance, improving clinically efficacy and perhaps conferring a vasculoprotective profile to chronically administered nitrates, thereby increasing our armamentarium against atherosclerosis.

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Combating Nitrate Tolerance: A Novel Endogenous Mechanism
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