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EDITORIAL COMMENT

Does Nitroglycerin Therapy Hit the Endothelium?*

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Nitrates are still widely used in the management of coronary artery disease (CAD) in patients with stable and unstable angina, acute myocardial infarction and congestive heart failure. The therapeutic efficacy of these nitrates is due to peripheral venous and arterial dilation that results in decreased myocardial oxygen consumption. Nitrates also dilate large coronary arteries and collaterals while having minimal or no effect on arteriolar tone. It is assumed that nitroglycerin (NTG) induces vasorelaxation by releasing the vasoactive principle nitric oxide (NO) via an enzymatic biotransformation step. Nitric oxide, an endothelium-derived relaxing factor, activates the target enzyme soluble guanylyl cyclase (sGC) and increases tissue levels of the second messenger cyclic guanosine monophosphate (cGMP). Cyclic GMP in turn activates a cGMP-dependent protein kinase which has been shown to mediate vasorelaxation via

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phosphorylation of proteins that regulate intracellular Ca^{2+} levels. Nitric oxide released from NTG may also beneficially influence the process of atherosclerosis by reducing neutrophil adhesion to the endothelium and by inhibiting the expression of adhesion molecules and platelet aggregation. Despite the beneficial hemodynamic profile and potential antiatherogenic effects of NTG, the efficacy of this kind of treatment in patients with CAD remains disappointing (1,2). In fact, a recent meta-analysis even indicates that the long-term use of nitrates may be deleterious for patients with ischemic heart disease (3).

NITRATE TOLERANCE AND CROSS-TOLERANCE

Although acute application of NTG exhibits high vasodilator and anti-ischemic efficacy, this activity is rapidly lost on long-term treatment due to the development of nitrate tolerance (4). The mechanisms underlying this phenomenon are likely to be multifactorial and may involve neurohormonal counter-regulatory mechanisms, impaired NTG biotransformation or changes intrinsic to the vasculature.

A phenomenon related to nitrate tolerance is cross-tolerance to other endothelium-dependent and -independent nitrovasodilators. This has been observed most commonly in situations in which NTG was administered long term in vivo in experimental animal models (4–6) and is not encountered in situations in which nitrate tolerance is induced by short-term exposure of vascular segments in vitro (7). It remains to be established whether nitrate therapy may adversely affect endothelial function in humans, a question that needs to be addressed since endothelial dysfunction has been shown to be a predictor of adverse long-term outcome in patients with CAD (8).

In this issue of the *Journal,* Gori et al. (9) present a paper that examines the effect of long-term NTG treatment on endothelial function of the forearm circulation of healthy volunteers. Endothelial function was assessed using strain gauge plethysmography. The NTG dose was 0.6 mg/h/d, which produces an average NTG concentration of about 0.1 μg/kg/min. The treatment period was 6 days and the study was designed in an investigator-blind parallel fashion. Flow responses of the brachial artery were studied in response to intra-arterial infusion of the endothelium-dependent vasodilator acetylcholine (ACh) and the NO synthase inhibitor N^G-monomethyl-L-arginine (L-NMMA). Continuous treatment with NTG patches for 6 days resulted in a marked inhibition of ACh-induced increases in forearm blood flow as compared to the control group without NTG pretreatment. Likewise, L-NMMA induced vasoconstriction was significantly blunted in volunteers treated with NTG. The lowest concentration of L-NMMA was even able to cause a paradoxical dilation. Based on the findings, the authors concluded that NTG treatment has an inhibitory effect on basal as well as on stimulated vascular NO-bioavailability and that this is, at least in part, due to abnormalities in NO synthase (NOS III) function.

Similar findings have previously been established in the coronary circulation of patients treated continuously with NTG for a 5-day period with NTG patches. Using ACh-induced vasoconstriction as a surrogate parameter for endothelial function in large coronary arteries, Caramori et al. (10) found that continuous treatment with NTG leads to enhanced ACh-induced vasoconstriction. The interpretation of the results of this study may be confounded by the fact that long-term NTG treatment also causes a hypersensitivity to vasoconstricting agonists (11) by activating the second messenger protein kinase C (PKC) and by stimulating the expression of endothelin-1 within the smooth muscle layer (12). Thus, by studying ACh-induced vasoconstriction in the coronary circulation, it may be difficult to differentiate whether this phenomenon is due to endothelial

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dysfunction or secondary to NTG-induced hyper-reactivity of the smooth muscle layer to constricting agonists, a question that is now addressed by the results of the Gori et al. (9) study.

MECHANISMS UNDERLYING NTG-INDUCED DIMINISHED VASCULAR NO BIOAVAILABILITY

The attenuated ACh forearm blood flow response and the attenuated L-NMMA response in healthy volunteers indicate diminished vascular NO bioavailability in response to long-term NTG treatment. As discussed by Gori et al. (9), several reasons may account for this phenomenon such as a decrease in the expression of NOS III, a dysfunctional NOS III due to intracellular L-arginine or tetrahydrobiopterin deficiency, increased vascular superoxide production or a desensitization of the NO target enzyme sGC at the smooth muscle level as suggested by Molina et al. (5). At first glance, it seems difficult to see how these quite different mechanisms may fit into one solid concept. A closer look, however, indicates that all of these findings may be explained by an NTG-induced increase in vascular superoxide production.

Evidence for a role of oxidative stress in tolerance and cross-tolerance was first provided by experimental studies showing that superoxide dismutase (SOD) was able to improve tolerance as well as cross-tolerance to ACh (4). Subsequently, in vitro as well as in vivo treatment with NTG has been shown to be associated with increased superoxide levels in endothelial as well as in smooth muscle cells (13,14). The stimulation of vascular superoxide production by NTG therapy may have several consequences.

First, increased superoxide production in endothelial and smooth muscle cells may inhibit the vasodilator potency of NTG simply by inactivating NO released from NTG during the biotransformation process.

Second, superoxide combines with NO in a diffusion-limited reaction that is about 10 times faster than the dismutation of superoxide by SOD. This reaction produces peroxynitrite, a compound with limited NO-like bioactivity thereby "shunting" NO away from its typical target functions such as vasodilation and platelet inhibition. In vitro as well as in vivo data indicate that NTG treatment increases vascular (15) and urinary nitrotyrosine levels (16), which can be considered as a marker of peroxynitrite-dependent oxidative damage. There is a growing body of evidence showing that increased vascular peroxynitrite formation may also have deleterious consequences for the function of the NOS III. As pointed out by Gori et al. (9), peroxynitrite is a strong stimulus for the oxidation of the NOS III cofactor tetrahydrobiopterin (BH_4) to dihydrobiopterin (BH_2) (17). The resulting intracellular BH_4 deficiency may lead to an uncoupling of NOS III (18). Thus, NTG therapy may switch NOS III from a NO to a superoxide-producing enzyme, which may further increase oxidative stress in vascular tissue in a positive feedback fashion. Indeed, an uncoupled NOS III has recently been demonstrated in an animal model of nitrate tolerance since an inhibitor of NOS III, L-NNA was able to significantly reduce vascular superoxide production in tolerant vessels (19). In addition, supplementation of NTG-treated rats with BH_4 was able to reverse NTG-induced endothelial dysfunction (20). A second mechanism of NOS III uncoupling may be intracellular depletion of L-arginine (21). Incubation of cultured endothelial cells or vascular tissue with NTG has been shown to reduce the vasodilator potency of NTG, to deplete intracellular L-arginine levels (22) and to stimulate endothelial cells to produce superoxide (23). Since endothelial superoxide production was blocked by NOS III inhibitors and tolerance was improved by L-arginine, the authors concluded that NTG-induced increases in superoxide production may be at least in part secondary to NOS III uncoupling due to L-arginine deficiency (23). It is not very likely that endothelial dysfunction is secondary to decreased expression of NOS III since recent experimental studies have shown that in the setting of tolerance, the enzyme is upregulated rather than downregulated (19).

Third, superoxide has recently been shown to be a potent stimulus for the activation of PKC in endothelial cells (24). In turn, PKC may phosphorylate NOS III, therefore leading to an inhibition of activity and inhibition of NO production by the enzyme (25).

Fourth, superoxide has also been shown to have a potent inhibitory effect on the activity of the NO downstream target, the sGC (26) and cGMP-dependent protein kinase action (13,27), which may partly explain the trend for a desensitization of the sodium nitroprusside dose response relationship for blood flow responses as described by Gori et al. (9).

The fact that NTG therapy indeed stimulates superoxide production in human tissue was recently shown by Sage et al. (28) in patients undergoing bypass surgery. However, the authors failed to demonstrate any cross-tolerance to endothelium dependent and independent vasodilators and in vitro modulation of vascular superoxide production did not modify the NTG-dose response relationship. In addition, they established a decreased tissue content of 1,2 glyceryl dinitrate in tolerant tissue, which was used as an argument to conclude that impaired NTG-biotransformation specifically accounts for tolerance and that endothelial function is preserved (28). Although differences in the vessel region studied (conductance vs. resistance vessels) as well as differences in the duration of NTG treatment (1 vs. 6 days) make it difficult to compare these two studies, the recent demonstration of cross-tolerance to endothelium-dependent vasodilators in coronary arteries (10) and the results of the present study (9) challenge the concept that impaired NTG biotransformation is the sole reason for nitrate tolerance.

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STRATEGIES TO PREVENT THE DEVELOPMENT OF TOLERANCE AND CROSS-TOLERANCE

Which strategy is the best to prevent the development of tolerance and cross-tolerance? The most widely accepted approach to prevent the tolerance phenomenon is a nitrate-free interval. Intermittent administration of NTG patches allowing a nitrate-free interval of 8 to 12 h has been shown to retain nitrate sensitivity, with the disadvantage of a lack of protection during this period. Another potential problem of a nitrate-free interval may also be the development of rebound ischemia. Treatment of experimental animals with a nitrate-free interval was not able to normalize endothelial dysfunction and hypersensitivity to vasoconstrictors (29). During the nitrate-free interval, the frequency of angina symptoms as well as of silent angina was significantly increased (30). By treating patients intermittently with NTG patches for a 5-day period, Azevedo et al. (31) recently showed that this kind of regimen may prevent the development of tolerance. Acute removal of the patch, however, increased the coronary vasomotor responses to ACh, suggesting that the rebound phenomena may be, at least in part, secondary to the development of endothelial dysfunction (31). These data clearly indicate that the phenomenon of NTG-induced endothelial dysfunction cannot be prevented by a nitrate-free interval.

If oxidative stress is important for tolerance and cross-tolerance, antioxidants or drugs, which are able to reduce oxidative stress within vascular tissue, should be able to positively influence both phenomena. Recent small studies in patients with CAD and heart failure indeed demonstrated that the development of tolerance is beneficially influenced by vitamin C (32,33), vitamin E (34) and angiotensin-converting enzyme (ACE) inhibitors (35,36). In addition, results from randomized trials in patients with heart failure (37) and acute myocardial infarction (1) indicate beneficial effects of nitrate therapy in combination with ACE inhibitors. It is tempting to speculate that the beneficial effects of ACE inhibitors are mediated at least in part due to the prevention of tolerance and cross-tolerance during NTG therapy.

WHAT QUESTIONS ARE RAISED BY THE PRESENT STUDY

Although the oxidative stress concept is an attractive one to explain the cross-tolerance phenomenon presented by Gori et al. (9), the presented data only indicate that basal as well as stimulated vascular NO bioavailability is disturbed in response to long-term NTG treatment. In particular, the question whether there is dysfunctional NOS III in vessels from NTG-treated patients has not yet been answered. For example, the authors discuss that L-NMMA can be used as a tool to study NOS III uncoupling, although studies with the NOS III enzyme indicated that L-NMMA, in contrast to N^G-nitro-L-arginine or its methylester, cannot inhibit superoxide production (38).

The concept of NOS III uncoupling as a mechanism of cross-tolerance requires further studies, which are able to demonstrate that NTG-induced endothelial dysfunction is improved by the modulation, e.g., of intracellular BH_4 levels with substances such as BH_4 itself, BH_4 precursors such as sepiapterin or substances that increase intracellular BH_4 levels like folic acid. In addition, studies have to be performed to show whether L-arginine supplementation or therapy with antioxidants such as vitamin C may prevent the development of endothelial dysfunction during long-term NTG treatment. It will also be interesting to see how patients with CAD and pre-existing endothelial dysfunction will respond to NTG therapy in this endothelial function model and whether the inhibition of oxidative stress by antioxidants is able to prevent tolerance as cross-tolerance.

Other clinically relevant questions are whether endothelial dysfunction occurs in response to intermittent treatment with mononitrates and dinitrates and whether it occurs with other nitrovasodilators such as molsidomine. What is the time course of endothelial dysfunction development and is there a dose dependency? Why does endothelial dysfunction develop in a vessel region such as the arterioles, where nitrates have little or no vasodilator potency (39)? Answers to all these questions would greatly enhance our understanding of mechanisms leading to tolerance and cross-tolerance.

SUMMARY AND CLINICAL IMPLICATIONS

In summary, there is mounting evidence that systemic therapy with NO via organic nitrates hits the endothelium in patients with CAD (10,31) and even in healthy control subjects (9). One mechanism contributing to this phenomenon may be a nitrate-induced stimulation of vascular superoxide production. Overall, this may represent a kind of biochemical baroreflex, in which the NTG-induced increases in vascular NO are diminished due to local degradation by superoxide. Treatment of patients with CAD with ACE inhibitors and HMG-CoA reductase inhibitors have been shown to improve endothelial dysfunction and simultaneously to improve prognosis. The present study indicates that nitrates cause endothelial dysfunction and a recent meta-analysis indicates that nitrates may worsen the prognosis in patients with ischemic heart disease (3). However, further studies are required to understand the precise nature of the mechanisms underlying NTG-induced endothelial dysfunction. These findings should be helpful to develop strategies for preventing these NTG-induced side effects.

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