Oxidative Inhibition of the Mitochondrial Aldehyde Dehydrogenase Promotes Nitroglycerin Tolerance in Human Blood Vessels

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Objectives
We tested the hypothesis of whether an inhibition of the nitroglycerin (GTN) bioactivating enzyme mitochondrial aldehyde dehydrogenase (ALDH-2) contributes to GTN tolerance in human blood vessels.

Background
The hemodynamic effects of GTN are rapidly blunted by the development of tolerance, a phenomenon associated with increased formation of reactive oxygen species (ROS). Recent studies suggest that ROS-induced inhibition of ALDH-2 accounts for tolerance in animal models.

Methods
Segments of surgically removed arteria mammaria and vena saphena from patients undergoing coronary bypass surgery were used to examine the vascular responsiveness to GTN and the endothelium-dependent vasodilator acetylcholine. The ALDH-2 activity and expression in these segments were assessed by the conversion of a benzaldehyde or its derivative to the benzoic acid metabolite and by Western blotting technique.

Results
In contrast to patients not treated with nitrates (n = 36), patients treated with GTN for 48 h (n = 14) before surgery showed tolerance to GTN and endothelial dysfunction in arterial and venous vessels. In vivo GTN tolerance was mimicked in vitro by incubation of nontolerant vessels with the ALDH-2 inhibitor benzoyl. In vivo GTN treatment decreased vascular aldehyde dehydrogenase activity compared with nontolerant vessels and decreased the expression of ALDH-2 in arterial tissue. Incubation of control venous vessels with GTN caused a significant attenuation of aldehyde dehydrogenase activity that was reversed by presence of the sulfhydryl group donor dithiothreitol.

Conclusions
Long-term GTN treatment induces tolerance and endothelial dysfunction in human vessels, associated with an inhibition and down-regulation of vascular ALDH-2. Thus, these findings extend results of previous animal studies to humans. (J Am Coll Cardiol 2007;50:2226–32) © 2007 by the American College of Cardiology Foundation
Some of these involved mechanisms, such as increased ROS production in A. mammaria, endothelial dysfunction, and an inhibition of the activity of the cyclic guanosine monophosphate-dependent kinase 1 (cGK-1) also have been demonstrated in patients with coronary artery disease (8). It remains to be established, however, whether in vivo treatment with GTN leads to an oxidative stress-induced inhibition of the GTN-metabolizing enzyme ALDH-2 in vascular tissue and whether GTN-induced endothelial dysfunction is associated with a change in the expression of the endothelial nitric oxide synthase gene (eNOS).

Methods

Study protocol. Patients were retrospectively assigned to a control group (CTR) without GTN treatment before surgery or to the GTN group being treated with continuous intravenous GTN treatment directly before surgery. Patients on a chronic oral nitrate therapy or intravenous nitrate therapy for <24 h were not included in the study. Exclusion criteria were concomitant treatment with antioxidants such as vitamin E and C. This study was approved by the local ethics committee of Mainz, Germany. Informed consent was given by all patients involved in the study.

The medication and characteristics of all patients participating in the study are summarized in Table 1. During surgery, discarded segments of the A. mammaria and V. saphena were collected. Additionally, in an initial series of patients (8) segments of A. mammaria, V. saphena, and A. radialis were collected to be compared in regard to their aldehyde dehydrogenase (ALDH) activity.

Vessel preparation and isometric tension studies. To test for vascular GTN sensitivity and for the determination of endothelial function, isolated rings of A. mammaria and V. saphena vessels were used in organ chamber experiments as described previously (8). Vessels were pre-constricted with the thromboxane analogue U46619 to achieve 30% to 50% of maximal (potassium chloride-induced) tone as described previously (8). Vessels were pre-constricted with endothelial function, isolated rings of A. mammaria and V. saphena were collected. Additionally, in an initial series of patients (8) segments of A. mammaria, V. saphena, and A. radialis were collected to be compared in regard to their aldehyde dehydrogenase (ALDH) activity.

Western blot analyses for eNOS and ALDH-2 expression. Frozen tissue was homogenized in liquid nitrogen, subjected to sodium dodecylsulfate-polycrylamide gel electrophoresis (SDS-PAGE) and subsequently blotted to nitrocellulose membranes (BioRad, Hercules, California). The blots were developed with a mouse monoclonal antibody to eNOS (dilution 1:1,000; BD Biosciences, San Jose, California) or a rabbit polyclonal Antibody to ALDH-2 (dilution 1:2,500; kindly provided from K.K. Ho, Purdue University, West Lafayette, Indiana [13]). Loading and transfer were normalized against alpha-actinin or to 2-hydroxy-3-nitrobenzoic acid, respectively, was measured in the incubation solution as described (10,12). One hundred microliters of each sample were subjected to high-performance liquid chromatography (HPLC) analysis. The half-maximal concentration (EC 50) value for each vascular activity experiment was obtained by logit-transformation. To compare different vessel types and treatment groups, a 1-way analysis of variance was employed. P values < 0.05 were considered locally significant with the

<table>
<thead>
<tr>
<th>Table 1</th>
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<tr>
<td></td>
<td>CTR</td>
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<tr>
<td>n</td>
<td>36</td>
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<tr>
<td>Age (yrs)</td>
<td>70 ± 1</td>
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<tr>
<td>GTN treatment period (h)</td>
<td>—</td>
</tr>
<tr>
<td>GTN dose (mg/h)</td>
<td>—</td>
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<tr>
<td>GTN dose (µg/kg/min)</td>
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<td>Gender, male/female</td>
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<td>Age &gt;60 yrs (%)</td>
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<td>Hyperlipidemia (%)</td>
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<td>Smoking (%)</td>
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<td>Diabetes (%)</td>
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<tr>
<td>Beta-blocker (%)</td>
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<td>ACE inhibitor (%)</td>
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<td>ASS/clopidogrel (%)</td>
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Values indicate the total number of patients/group or mean ± SEM.

Abbreviations and Acronyms

ALDH-2 = mitochondrial aldehyde dehydrogenase
DTT = dithiothreitol
EC50 = half-maximal concentration
eNOS = endothelial nitric oxide synthase
GTN = glycerol trinitrate, nitroglycerin
ROS = reactive oxygen species
ACE = angiotensin-converting enzyme; ASS = acetylsalicylic acid; AT1 = angiotensin 1; CAD = coronary artery disease; CTR = control group without in vivo nitroglycerin treatment; CVD = cardiovascular disease; GTN = vessels from patients with intravenous nitroglycerin treatment; VD = vessel disease.
patient being the unit of analysis. Owing to very limited and variable amounts of waste bypass material, the statistical analysis of different treatment groups was performed for each individual experimental setting and not in a paired manner. If more than 1 vessel segment/patient was analyzed, the mean value was used for further statistical analysis. The total number of patients and vessel segments are reported in the figure legends.

**Results**

**Studies of vascular reactivity.** Vascular relaxations to GTN and acetylcholine: GTN treatment in vivo caused a significant shift to the right of the GTN dose-response relationship in A. mammaria and V. saphena, compatible with the development of tolerance. Likewise, in vitro incubation of these vessels the ALDH-2 inhibitor benomyl shifted the GTN dose-response curve to a comparable degree to the right (Fig. 1, Table 2). In both vessel types studied, GTN in vivo caused a significant degree of endothelial dysfunction in A. mammaria and V. saphena segments (Table 2).

**Vascular ALDH activity.** Intravenous GTN treatment inhibited the ALDH activity. In vitro incubation with the ALDH-2 inhibitor benomyl suppressed ALDH activity in vessels of patients without GTN pretreatment by approximately 75% (Fig. 2). Likewise, in vitro incubation of vessels of patients without GTN pretreatment with GTN attenuated ALDH activity markedly (Fig. 2). The use of the alternative substrate, benzaldehyde, to test for dehydrogenase activity of ALDH revealed that GTN strongly inhibited ALDH activity that could be prevented by co-incubation with the dithiol compound dithiothreitol (Fig. 3), pointing to a redox-sensitive regulation of the human ALDH enzyme system. Further analysis of ALDH activity from A. mammaria, A. radialis, and V. saphena bypass graft material revealed that the weight-normalized activity was highest in A. mammaria, followed by V. saphena and radial arteries (Table 3).

**Effects of GTN treatment on eNOS and ALDH-2 protein expression.** The GTN treatment led to a significant decrease in protein expression of the eNOS and ALDH-2 by about 40% in A. mammaria (Figs. 4 and 5). No change in eNOS expression was detected in V. saphena segments in response to GTN in vivo treatment.

**Discussion**

With the present study we show that in vivo GTN treatment for approximately 48 h causes tolerance in venous and arterial bypass vessels that is associated with an inhibition of vascular mitochondrial ALDH activity, the GTN bioactivating enzyme. Tolerance in A. mammaria was associated with a significant decrease in ALDH-2 expression. In control vessels, the inhibition of ALDH-2 activity could be mimicked in vitro by incubation with GTN or the ALDH-2 inhibitor benomyl. The antioxidant DTT restored ALDH-2 activity, suggesting that ROS are likely causally involved in the inhibition of the enzyme in response to GTN therapy.

Nitrates are still widely used for the treatment of acute coronary syndrome and acute and chronic congestive heart failure. The anti-ischemic and vasodilator properties of GTN, however, are rapidly blunted, owing to the development of nitrate tolerance that occurs within 1 to 3 days of continuous treatment. The 2 mechanisms accounting for this involve mainly an increase in oxidative stress (3,14) and an impairment of the ALDH-2 mediated GTN biotransformation process within the vascular tissue itself (4). Recently, we were able to demonstrate that, similar to results obtained in experimental animals (3,14,15), in vivo GTN treatment averaging 0.5 μg/kg/min for 48 h causes a marked

![Figure 1: Effects of In Vivo Nitroglycerin Treatment and In Vitro ALDH Inhibition on Nitroglycerin Reactivity in Human Bypass Vessels](image)
degree of tolerance in humans (8). This was associated with endothelial dysfunction and an inhibition of the activity of the cGK-I, the enzyme being of utmost importance for GTN-induced vasorelaxation (8).

We proposed, as a predominant mechanism for tolerance and endothelial dysfunction, that GTN-induced increases in the formation of ROS account for both phenomena. The results of the present studies go along with this concept, because we found that GTN treatment for 48 h in a concentration averaging 0.5 \( \mu \text{g/kg/min} \) caused a comparable degree of tolerance and endothelial dysfunction in patients undergoing bypass surgery. Impaired biotransformation as well as oxidative stress contribute to tolerance. Recently, the group of Stamler...
et al. (4) proposed that the enzyme ALDH-2, located in mitochondria, represents the nitrate reductase responsible for bioactivation of GTN. This assumption was based on in vitro and in vivo experiments showing that more or less specific inhibitors of the enzyme (4,16), such as cyanamide, chloral hydrate, as well as acetaldehyde, shifted the GTN but not the sodium nitroprusside dose response curve to the right, as in vitro incubations of vascular tissue with high-dose GTN (in vitro tolerance). Inhibitors blocked the GTN-induced increases in cGMP in isolated aortic rings. Recently, these studies were extended to studies in ALDH-2 -/- mice supporting the initial findings (5). Importantly, GTN bioactivation by ALDH-2 and subsequent tolerance phenomena might be limited to lower, therapeutically relevant GTN concentrations (5), and the ALDH-2 does not seem to be involved in the biotransformation process of mono- and dinitrates (1).

In a recent study, we were able to demonstrate that the impaired GTN biotransformation concept as well as the oxidative stress concept are closely related to each other (10). With these studies we found that acute incubation of mitochondria in vitro with GTN leads to an increase in ROS production associated with an inhibition of the mitochondrial ALDH-2 (10). These findings were extended by our in vivo observations demonstrating that GTN treatment of Wistar rats for a 3-day period increased mitochondrial ROS production and inhibited simultaneously the activity of the enzyme (12). In addition, endothelial cells without mitochondria showed no increase in cGMP levels in response to GTN as compared with endothelial cells with mitochondria (10). Thus, the current unifying concept of tolerance proposes that in vivo treatment with GTN increases oxidative stress within mitochondria at complex-I of the respiratory chain (9), all of which might cause oxidation of critical sulphydryl (SH)-groups near the active center of the ALDH, thereby leading to an inhibition of the enzyme (6). This hypothesis was further strengthened by recent studies in mice with heterozygous manganese superoxide dismutase knockout, which have (owing to partial deficiency in this antioxidative protein) increased mitochondrial oxidative stress and simultaneously showed increased susceptibility for the development of nitrate tolerance (12).

To test whether this concept might also apply to human vessels, tissue from the A. mammaria and V. saphena of patients undergoing bypass surgery were incubated with the sensitive and specific inhibitor of the ALDH-2 benomyl. Interestingly, the rightward shift in the GTN dose-response relationship in vascular tissue from patients without GTN pretreatment was almost identical as compared with the one observed in vessels from intravenously GTN-treated patients. In parallel, we found a significant inhibition of the activity of the ALDH-2. Likewise, incubation of both arteries and veins from control group patients with GTN (in vitro tolerance) resulted in a significant inhibition of the activity of the enzyme. Thus, it is conceivable to conclude that in vivo tolerance induced with clinically relevant concentrations of GTN is at least in part secondary to an inhibition of the ALDH-2. This concept is further supported by recent clinical trials indicating that a point mutation in ALDH-2 (leading to an inactive enzyme), which appears in high prevalence in Asian people, decreases GTN vasodilator potency (7).

With the present study we found no difference in GTN potency between human V. saphena and A. mammaria in nontolerant tissue (Table 2). The 3 vessel types studied differed with respect to their ALDH activity being the highest in A. mammaria and the lowest in the radial artery (Table 3). These findings suggest that GTN bioactivation might differ throughout the body without a general preference for either arteries or veins. Nevertheless, we cannot exclude that, for example, the more traumatic preparation of the vein and the radial artery might have decreased to some extent their ALDH-activity. In addition, we do not know whether veins in situ react more sensitively to GTN compared with excised veins.

To test for redox sensitivity of ALDH-2 activity as a possible mechanism for its GTN-induced inhibition, human V. saphena was incubated with GTN in presence or absence of the sulphhydryl-group donor DTT. The finding

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**Table 3** ALDH Activity in Human Bypass Graft Vessels Without Prior GTN Treatment

<table>
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<tr>
<th>Tissue</th>
<th>Benzoic Acid [µmol/l/mg]</th>
<th>SEM</th>
<th>n</th>
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<tr>
<td>A. mammaria</td>
<td>9.8</td>
<td>0.8</td>
<td>15</td>
</tr>
<tr>
<td>A. radialis</td>
<td>3.6</td>
<td>0.3</td>
<td>10</td>
</tr>
<tr>
<td>V. saphena</td>
<td>4.3</td>
<td>0.3</td>
<td>8</td>
</tr>
</tbody>
</table>

Conversion of benzaldehyde (400 µmol/l) to benzoic acid was measured by high-performance liquid chromatography. Mean data ± SEM; n indicates number of patients/group from 27 to 41 measurements/group.

ALDH = mitochondrial aldehyde dehydrogenase; GTN = nitroglycerin.
that DTT completely restored ALDH-2 activity further substantiates the concept of an oxidative stress-induced inhibition of the enzyme. Likewise, Sage et al. (17) demonstrated impaired GTN biotransformation in vessels from patients undergoing bypass surgery, who were treated with GTN (0.15 μg/kg/min) for 24 h and simultaneously increased oxidative stress as assessed by lucigenin-enhanced chemiluminescence.

In the present study, in vivo GTN treatment not only inhibited ALDH-2 activity but also decreased its expression in the A. mammaria substantially by approximately 40%. This finding goes along with a more recent publication where we could establish in an animal model of nitrate tolerance that GTN therapy markedly down-regulates ALDH-2 expression in rat aorta (6).

Mechanisms underlying GTN-induced endothelial dysfunction. As shown repeatedly, tolerance is associated with a marked degree of cross tolerance to the endothelium-dependent vasodilator acetylcholine. Previously, we and others have proposed that this phenomenon is rather secondary to a GTN-induced increase in ROS production throughout the vessel wall, leading to enhanced NO breakdown and subsequently to an increase in endothelial peroxynitrite production (18). Peroxynitrite per se is a strong stimulus for the oxidation of the eNOS cofactor BH₄ to the BH₃ radical or BH₂ (19), all of which might lead to an uncoupling of the enzyme, meaning that the enzyme produces superoxide instead of NO. Indeed, this uncoupling reaction has been confirmed previously in an animal model of nitrate tolerance (14).

The results of animal studies also indicated that endothelial dysfunction was associated with an up-regulation rather than a down-regulation of eNOS (6). The results of the present study show that GTN treatment decreases the expression of the enzyme in human A. mammaria but did not significantly change the expression in veins. Thus, on the basis of these heterogenous results it remains to be

**Figure 4** Effects of Nitroglycerin Treatment on eNOS Expression in Human Bypass Vessels

Nitroglycerin (GTN) treatment led to a significant decrease in endothelial nitric oxide synthase (eNOS) expression in A. mammaria, whereas eNOS expression in V. saphena was not affected. Data are mean ± SEM from n = 9 to 11 experiments/group. *p < 0.05 versus without GTN treatment (CTR).

**Figure 5** Effects of Nitroglycerin Treatment on ALDH-2 Expression

Nitroglycerin (GTN) treatment led to a significant decrease in mitochondrial aldehyde dehydrogenase (ALDH-2) expression in A. mammaria compared with control subjects. Data are mean ± SEM from n = 10 to 12 experiments/group. *p < 0.05 versus without GTN treatment (CTR).
determined whether changes in eNOS expression do contribute to endothelial dysfunction at all.

Summary and clinical implications. The results of the present studies clearly show for the first time that in vivo treatment of patients with established coronary artery disease with GTN for more than 24 h causes tolerance that was associated with a marked inhibition of the ALDH-2 and a significant decrease in the expression of the enzyme. The activity of the enzyme could be restored by the dithiol compound DTT. Thus, the experimental concept that GTN induces oxidative stress and leads to an inhibition of the GTN metabolizing enzyme (10) also seems to hold true in the clinical setting. Future studies have to demonstrate whether the use of, for example, mitochondria-specific antioxidants might help to prevent the development of endothelial dysfunction and tolerance in response to GTN in patients or whether organic nitrates, being able to stimulate antioxidant pathways such as heme oxygenase (6), might be the preferable nitrate in the future treatment of patients with stable/unstable coronary artery disease or chronic congestive heart failure.

Acknowledgments
The authors thank Jörg Schreiner and Hartwig Wiebolt for expert technical assistance.

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REFERENCES