Monitoring White Blood Cell Mitochondrial Aldehyde Dehydrogenase Activity: Implications for Nitrate Therapy in Humans

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ABSTRACT

Recent animal data suggest that reduced lipoic acid (LA) prevents oxidative inhibition of the nitrate bioactivating enzyme, the mitochondrial aldehyde dehydrogenase (ALDH-2), and that pentaerythritol tetranitrate (PETN) does not induce nitrate tolerance because of its intrinsic antioxidative properties, thereby preserving ALDH-2 activity. We sought to determine whether ALDH-2 activity in circulating white blood cells (WBCs) can be used to monitor nitrate tolerance and whether LA can prevent nitroglycerin tachyphylaxis in humans. Eight healthy male volunteers received, in randomized order, a single dose of glyceryl trinitrate (GTN; 0.8 mg), PETN (80 mg), or GTN plus LA (600 mg) orally. GTN (30 min) and PETN (120 min) administration lead to a comparable dilation of the brachial artery (15 ± 1%). In contrast to PETN, acute GTN treatment resulted in a 60% decrease in WBC ALDH-2 activity (high-performance liquid chromatography), 30% reduction of nitrate bioactivation, and 25% decrease in serum antioxidant capacity (fluorescence assay), which all were prevented by pretreatment with LA. Mechanistic studies in rats identified oxidative stress, ALDH-2 inactivation, and vascular dysfunction as common features in acute and chronic nitrate tolerance. Treatment with GTN, but not PETN, acutely inhibits ALDH-2 activity and nitrate bioactivation in healthy volunteers. These effects were prevented by LA pretreatment, emphasizing the role of oxidative stress-triggered ALDH-2 dysfunction. Assessment of WBC ALDH-2 activity could be used as an easily accessible marker for the detection of nitroglycerin-induced tachyphylaxis in humans and may be of high clinical interest because recent data suggest that ALDH-2 activity correlates with protection from ischemic heart damage in infarct models.

The vascular endothelium, particularly its major product, nitric oxide (NO), is of critical importance in modulating nutrient provisioning, blood flow, vascular inflammatory phenomena, thrombus formation, and cell proliferation and, therefore, is a central constituent of the regulatory instruments of the vascular system (Arnold et al., 1977; Radomski et al., 1987). Dysfunction of this complex biochemical balance (i.e., “endothelial dysfunction”) has been identified as a hallmark of most cardiovascular diseases (Cai and Harrison, 2000). Its characteristic features include vascular oxidative stress, decreased NO bioavailability, and/or impaired activity (“uncoupling”) of endothelial NO synthase (Münzel et al., 2005b). These observations have important clinical implica-

ABBREVIATIONS: NO, nitric oxide; GTN, glyceryl trinitrate (nitroglycerin); PETN, pentaerythritol tetranitrate; ALDH-2, mitochondrial aldehyde dehydrogenase; ALDH, the sum of all aldehyde dehydrogenase isoforms; HO-1, heme oxygenase-1; WBC, white blood cell; LA, lipoic acid; DHE, dihydroethidine; Tx, thromboxane; HPLC, high-performance liquid chromatography; ACh, acetylcholine; ROS, reactive oxygen species; L-012, 8-amino-5-chloro-7-phenylpyrido[3,4-d]pyridazine-1,4-(2H,3H)-dione sodium salt; ECL, enhanced chemiluminescence; DTT, dithiothreitol; PBMC, peripheral blood mononuclear cell.
tions because patients with an impaired endothelial function have a higher risk of cardiovascular events (Schächinger et al., 2000).

Exogenous supplementation of NO seems to be an attractive pharmacological strategy to overcome endothelial (vascular) dysfunction in the setting of cardiovascular disease (Anderson et al., 1994). The most prominent representatives of this class of vasodilators are sodium nitroprusside, S-nitroso-N-acetyl-penicillamine, molsidomine (active metabolite 3-morpholino-sydnonimine), and organic nitrates (such as glyceryl trinitrate, pentaerythritol tetranitrate, or isosor- bare-5-mononitrate) (Gryglewski et al., 1992). It is unfortunate that the chronic use of these drugs is hampered by serious side effects, such as uncontrolled hypotension and cyanide intoxication, as known for sodium nitroprusside (Pearl et al., 1984), and a paradoxical increase in oxidative damage induced by 3-morpholino-sydnonimine (Berkenboom et al., 1990) and organic nitrates such as glyceryl trinitrate (nitroglycerin) (GTN) and isosorbide-5-mononitrate (Münzel et al., 2005a; Daiber et al., 2008), leading to endothelial dysfunction.

More recently, we and others were able to demonstrate that not all nitrates are the same with respect to their bioactivating process and their capacity to stimulate vascular superoxide production (Daiber et al., 2004b, 2008). For instance, we demonstrated that the nitrate pentaerythritol tetranitrate (PETN), although still undergoing mitochondrial activation by the ALDH-2 (Wenzel et al., 2007b), does not induce tolerance and does not stimulate vascular superoxide production in a rat model of nitrate tolerance, two phenomena that probably follow the activation of the antioxidant enzyme heme oxygenase-1 (HO-1) by PETN (Wenzel et al., 2007c). Mitochondrial reactive oxygen species formation was identified as a hallmark of GTN-induced nitrate tolerance (Sydow et al., 2004; Daiber et al., 2005; Esplugues et al., 2006), leading to oxidative ALDH-2 inhibition that could be prevented or reversed in an experimental rat model by (di-hydro)lipoic acid (Wenzel et al., 2007a). Recently, a Polish group reproduced our previous findings and demonstrated prevention of nitrate tolerance by lipoic acid in a more clinically relevant setting (Dudek et al., 2008).

Beyond the role of ALDH-2 for organic nitrate bioactivation, a very recent publication in Science (Chen et al., 2008) provides strong evidence that ALDH-2 activation (e.g., by ethanol and protein kinase Cε stimulation) is associated with a reduction of ischemic damage to the heart; vice versa, inhibition of ALDH-2 (e.g., by cyanamide or nitroglycerin) increases infarct size in an experimental model of myocardial infarction. In view of this new clinical implication of ALDH-2 in ischemic heart diseases, reliable measurement of ALDH-2 activity in white blood cells (WBCs) seems to be an attractive marker to assess potential ischemic damage to the heart.

With the present study, we aimed to test the effect of a single nitrate administration on human WBC ALDH-2 activity and to investigate the differences between GTN and PETN in this context and the protective effect of lipoic acid on human nitrate tachyphylaxis. Finally, we tested the hypothesis that characterization of WBC ALDH-2 activity can be used as an easily accessible surrogate marker for the development of vascular nitrate tolerance.

Materials and Methods

Volunteer Treatment Protocol. Eight healthy nonsmoking white male volunteers were assigned to take a single oral dose of GTN (0.8 mg sublingual), PETN (80 mg), or GTN plus pretreatment of α-lipoic acid (LA; 600 mg, 15 min in advance). Blood was sampled from an antecubital vein immediately before nitrate treatment and after maximal plasma concentrations were reached (30 min for the GTN di- and mononitrate metabolites and GTN-LA (ALIUD PHARMA GmbH, personal communication) and 120 min for the PETN dinitrate metabolite). For details on maximal plasma levels, see supplemental data. Serum for nitrite, nitrate, thromboxane, and S-nitrosoproteins (serum gel monovette, 10 min, 3600g) was snap-frozen in liquid nitrogen and stored at −80°C until analysis. Treatment of volunteers was in accordance with the Declaration of Hel- sinki and was granted by the local institutional Ethics Committee.

Nitrate-Dependent Dilation of the Brachial Artery. Organic nitrate-mediated dilation of the brachial artery was noninvasively examined by two-dimensional high-resolution ultrasonic imaging as described previously (Warnholtz et al., 2005) using a 7.5- to 12-MHz linear array transducer and HDI5000 ultrasound system (Philips, Hamburg, Germany). The diameter and blood flow at the level of the brachial artery were measured at rest and at the time points indicated above after GTN or PETN administration. The brachial artery segment diameter was determined by commercially available edge detection software (Brachial Analyzer; Medical Imaging Application, Iowa City, IA).

Measurement of Nitric Oxide Endproducts. Nitrite and nitrate in serum were measured simultaneously by gas chromatography-mass spectrometry as described elsewhere (Tsikas, 2000). Total plasma S-nitrosoproteins were measured by gas chromatography-mass spectrometry as described previously (Tsikas et al., 1999). All measurements of NOx species were performed in the laboratory of D. Tsikas (Hannover, Germany).

Isolation of WBCs. Leukocytes were isolated as described previously (Daiber et al., 2004a) and summarized in supplemental data. Total blood cell count and the purity of the fractions were evaluated using an automated approach using a hematology analyser KY-21N (Sysmex Europe GmbH, Norderstedt, Germany). All data were later normalized on cell number and protein content. The typical constitution of the blood cell fractions is presented in Supplemental Table 1.

Serum Antioxidative Capacity. Serum was prepared by centrifugation of whole blood in a serum-gel monovette at 3600g for 10 min. Freshly prepared serum was kept on ice and was used within 30 min. Either 25 or 50% serum was used to add phosphate-buffered saline buffer, which contained 5 μM DHE and 0.5 mM hypoxanthine. The assay was started by addition of 50 μM xanthine oxidase, and the fluorescence signal (excitation, 520 nm; emission, 610 nm) was mea- sured by using a Twinkle LB 970 fluorescence microplate reader from Berthold Technologies (Bad Wildbad, Germany) for 15 min. The assay determined the antioxidative capacity of serum by the decrease in xanthine oxidase-triggered superoxide formation generating DHE fluorescence; it allowed the determination of antioxidative capacity/properties of various drugs or fluids.

Thromboxane Formation. Serum was prepared as described above and snap-frozen. After thawing, thromboxane (TXa2, the stable hydration product of TXa1, was measured in serum using a commercially available TXa2 enzyme immunoassay kit according to the manufacturer’s instructions (Assay Designs, Ann Arbor, MI).

Nitrate Bioactivation. We recently have developed a fluorescence-based method to analyze nitrate bioactivation by using a dyesl-tagged PETN metabolite in collaboration with Lehmann and coworkers (University of Jena, Jena, Germany) (Wenzel et al., 2007b). It is important that the limits of detection and quantification were 50 and 100 nM, respectively. Representative chromatograms for this assay are shown in Supplemental Fig. 1.
Animals and in Vivo Treatment. To assess the chronic effects of GTN treatment, male Wistar rats (250 g) (Charles River, Margate, Kent, UK) were equipped with micro-osmotic pumps model 2001 from Alzet (Cupertino, CA) containing 450 mM GTN (average infusion rate, 6.6 μg/kg/min; solvent (ethanol)-containing minipumps served as control) or at a dilution of 1:2 or 1:8 [GTN/ethanol (v/v)] for 4 days. GTN-infusion was performed as described previously (Sydowet al., 2004). To determine the acute effects of GTN administration, some rats received a single oral GTN dose (15 μg/kg) by allowing the rats to lick 20 μl of a sweetened GTN solution and were sacrificed 30 min later. Animals were killed by exsanguination under isoflurane anesthesia (5% inhalant in room air). All animal treatment was in accordance with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996) and was granted by the Ethics Committee of the University Hospital Mainz.

ALDH-2 Activity in Isolated WBCs, Rat Aortic Tissue, and Isolated Rat Cardiac Mitochondria. Assays were performed using an HPLC-based method as described in previous publications (Daiber et al., 2004b; Wenzel et al., 2007b) and in supplemental data. For the assay of ALDH-2 activity in humans, WBCs were isolated as described above and in supplemental data.

Isometric Tension Studies in Rat Aorta. Vasodilator responses to the endothelium-dependent vasodilator acetylcholine (ACh) and the endothelium-independent GTN were assessed with endothelium-intact isolated rat aortic rings mounted for isometric tension recordings in organ chambers as described previously (Münzel et al., 1995; Daiber et al., 2004b). The rat aorta was preconstricted with phenylephrine (0.5–1 μM) to cause 60 to 70% of the tone induced by a KCl bolus.

Detection of Oxidative Stress in Rat Mitochondria and Isolated Aorta. Isolated mitochondria were prepared from rat hearts according to a previously published protocol, and reactive oxygen species (ROS) formation was detected by L-012 (100 μM) ECL as recently described (Daiber et al., 2004b) and detailed in supplemental data.

Statistical Analysis. Results are expressed as mean ± S.E.M. One-way analysis of variance (with Bonferroni’s or Dunn’s correction for comparison of multiple means) was used for comparisons of vasodilator potency and efficacy, ROS detection by chemiluminescence, ALDH-2 activity, nitrate bioactivation, serum antioxidative capacity, thromboxane levels, vasodilatory potency (brachial artery diameter), plasma S-nitrosoproteins, and serum nitrite and nitrate levels. The EC_{50} value for each experiment was obtained by log-transformation. Values of p < 0.05 were considered significant.

Results

Nitrate-Induced Dilation of the Brachial Artery, S-Nitrosylation of Plasma Proteins, and Serum Nitrite and Nitrate Formation. As shown in Fig. 1A, dilation in response to PETN peaked at 2 h and then continuously declined until the initial diameter of the brachial artery was reached at 24 h post-PETN intake. The dilation of the brachial artery in response to GTN (NMD) was assessed 30 min after sublingual administration of the drug and was not statistically different from the PETN response at 120 min. Representative two-dimensional images of the brachial artery diameters at rest and the time point of peak plasma dilation are shown in Fig. 1B.

Fig. 1. Effect of in vivo challenges with GTN or PETN on hemodynamic parameters in human volunteers. A, peripheral vascular function was determined before (basal) and after oral treatment with GTN (0.8 mg) or PETN (80 mg). B, representative two-dimensional images of the brachial artery at rest and after the application of the drug. C, changes in systolic (Sys) and diastolic (Dia) blood pressure and heart rate (HR) elicited by PETN (2 h) or GTN (30 min), bpm, beats per minute. The data are mean ± S.E.M. of four to eight volunteers/group. *, p < 0.05 versus prior treatment.
levels (120-min PETN, 30-min GTN) are shown in Fig. 1B. The decrease in blood pressure and increase in heart rate observed for both nitrates were not significantly different, indicating equipotent hemodynamic effects by the employed nitrate doses and the chosen time points (Fig. 1C). Measurement of S-nitroso-BSA, nitrate, and nitrite and detailed considerations on organic nitrate plasma levels are provided in Supplemental Fig. 2.

**Effect of Nitrate Treatment on ALDH-2 Activity in Human White Blood Cells.** In cells from buffy coat fractions (WBCs), 53% of the total ALDH activity could be blocked by daidzin, a specific inhibitor of ALDH-2 isoform (Fig. 2A). In vitro incubation of isolated WBCs with GTN (4.5 μM) resulted in a profound inhibition of enzyme activity (19% compared with untreated control), which could be partially restored (55% of untreated control) by coincubation with the sulphydryl donor dithiothreitol (DTT). In vivo, a single oral dose of GTN caused a decrease in WBC ALDH activity to 33% of the untreated control. It is important that the specific ALDH-2 inhibitor daidzin caused no further reduction in ALDH activity, indicating that the ALDH-2 isoform was already blocked completely by GTN in vivo treatment. Again, DTT reduced the effect of GTN on enzyme activity and caused a 2.2-fold increase in product formation. In vitro bolus incubation of isolated WBCs with PETN (4.5 μM) caused a decrease in enzyme activity to 27% of control. In contrast, oral PETN treatment did not impair ALDH-2 activity. Both DTT and the physiological thiol compound dihydrolipoic acid reversed the GTN-mediated inhibition of ALDH-2 activity in isolated WBCs (Fig. 2B). ALDH activity was also measured in whole blood, but the contribution of the ALDH-2 isoform was less than 30%, suggesting that the majority of blood cells contain other ALDH isoforms (Supplemental Fig. 5).

**Effects of Acute Oral Nitrate Treatment on WBC ALDH-2 Activity, Nitrate Bioactivation, Serum Antioxidative Capacity, and Thromboxane Levels in Humans.** In vivo, acute sublingual GTN treatment caused a reproducible, significant decrease in human WBC ALDH-2 activity (Fig. 3A). Oral pretreatment with lipoic acid 15 min before GTN prevented this effect. In contrast, a single dose of PETN caused no significant change in WBC ALDH-2 activity. Organic nitrate bioactivation capacity in isolated WBCs was inhibited to 74% of control (p < 0.05) after a single sublingual GTN administration. This was prevented by lipoic acid cotreatment (92% of control). No significant change (>86%) was observed in response to oral PETN challenges (Fig. 3B). In accordance with the observed oxidative inhibition of ALDH-2 activity, oral GTN administration caused a decrease of 15% in serum antioxidative capacity (Fig. 3C). Cotreatment with lipoic acid increased this parameter by 9%. Oral PETN administration did not modify serum antioxidative capacity (p < 0.05 for the comparison between PETN and GTN-LA versus GTN alone). Acute GTN treatment caused a

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**Fig. 2.** Effect of in vitro and in vivo treatment with GTN on WBC ALDH-2 activity in human volunteers. A, ALDH-2 activity in isolated WBCs was assessed upon treatment of WBCs in vitro with daidzin (200 μM), GTN (4.5 μM) with or without DTT (1 mM), benomyl (100 μM), or PETN (5 μM). In vivo, GTN (0.8 mg) and PETN (80 mg) were orally administered. The data are mean ± S.E.M. of six volunteers/group (in vivo) and six to 18 independent experiments (in vitro). B, restoration of GTN (4.5 μM)-impaired WBC ALDH-2 activity by in vitro incubation with reduced lipoic acid (dihydrolipoic acid (DHLA)) or DTT. The data are mean ± S.E.M. of three to five independent measurements. C, representative chromatograms before (black) and after (gray) GTN treatment of a volunteer. *, p < 0.05 versus control group; #, p < 0.05 versus GTN in vitro-treated group; §, p < 0.05 versus GTN in vivo-treated group; $, p < 0.05 versus PETN in vivo-treated group. All data obtained with separated WBC fraction were normalized to protein content; adjustment for cell number produced similar effects (Supplemental Figs. 3 and 4).
significant 73% increase in thromboxane levels, which was almost completely normalized by lipoic acid cotreatment (increase by only 13%). In contrast, PETN treatment did not modify plasma thromboxane levels, although a clear tendency for an increase was observed (Fig. 3D).

**Effect of Acute Oral GTN Treatment in Rats.** Rats were treated with a single oral dose of GTN (15 μg/kg) equivalent to the one used in the volunteers (the rats were allowed to lick a sweetened GTN solution that may be equal to sublingual treatment in humans); aortic ring segments taken within 30 min from these animals showed a small but significant degree of endothelial dysfunction (Fig. 4A) and GTN-tachyphylaxis as indicated by the impaired vasodilatory potency of GTN (Fig. 4B; Supplemental Table 2). Acute oral GTN therapy increased mitochondrial oxidative stress by 36% (Fig. 4C) and decreased total aortic ALDH activity by 28% (Fig. 4D), whereas mitochondrial and WBC ALDH activity were reduced by 17% (Fig. 4E) and 15% (Fig. 4F), respectively.

**Dose-Dependent Effect of Chronic GTN Treatment in Rats.** To demonstrate whether a correlation between ALDH-2 activity and degree of nitrate tolerance exists, rats were treated via infusion of GTN (0, 0.73, 2.2, or 6.6 μg/kg/min) or solvent (ethanol). Chronic (4 days) GTN treatment impaired responses to ACh in isolated rat aorta at all doses tested (Fig. 5A). Chronic infusions of increasing GTN doses induced higher degree of vascular tolerance to GTN (Fig. 5B; Supplemental Table 2), increased mitochondrial ROS formation, and inhibited mitochondrial ALDH-2 activity (Fig. 5, C and D). It is important that rat vascular tolerance showed a dose-dependent increase that paralleled the decline observed in vascular and WBC ALDH (Fig. 5, E and F; for normalization on cells, see Supplemental Fig. 6). At the same time, malate/glutamate-stimulated mitochondrial ROS formation was dose-dependently increased in response to all GTN doses tested (Supplemental Fig. 7). The direct connection between ALDH activity and GTN potency was further supported by direct correlation plots of WBCs and vascular ALDH activity and mitochondrial ALDH-2 activity versus GTN potency for all tested animals (Fig. 6). From these data, it is evident that ALDH activities in all samples decrease with increasing GTN dose and are correlated with a decrease in GTN potency.

**Discussion**

In the present study, we show that administration of a single sublingual GTN dose-significantly decreased WBC ALDH-2 activity and WBC nitrate bioactivation capacity, whereas an equipotent dose of PETN does not. It is important that the inhibitory effect of GTN on WBC-ALDH-2 activity could be prevented by LA, confirming animal data showing the protective role of LA for GTN tolerance. Moreover, we show in an animal model that the dose-dependent development of nitrate tolerance to GTN parallels GTN-induced mitochondrial oxidative stress and inactivation of ALDH-2 in mitochondria, vascular tissue, and WBCs (Fig. 5). In summary, assessment of WBC ALDH-2 activity could represent an easily accessible diagnostic window to nitrate-induced side effects in humans in vivo.

In healthy volunteers, a single sublingual dosage of GTN decreased the antioxidative capacity of serum and increased thromboxane levels, which is compatible with a pro-oxidative
and prothrombotic condition (Fig. 7). This increase in thromboxane formation is indeed best explained by the fact that GTN-derived peroxynitrite formation may activate the enzyme cyclooxygenase (via the peroxide tone) (Schildknecht et al., 2005, 2008), whereas vascular (D), mitochondrial (E), and WBC (F) ALDH-2 activities were assessed by an HPLC method. The data are mean ± S.E.M. of six to seven animals/group. Mitochondrial ROS formation (C) was measured by ECL, whereas vascular (D), mitochondrial (E), and WBC (F) ALDH-2 activities were assessed by an HPLC method. The data are mean ± S.E.M. of six to seven animals/group.

The information gathered from the measurement of WBC ALDH-2 can be used to develop therapies that prevent these side effects. In the present study, in accordance with previous animal studies (Wenzel et al., 2007a), ALDH-2 inhibition and other evidence of oxidative stress were significantly attenuated by pretreatment with lipoic acid, which suggests that this drug could be suitable for cotherapy with GTN in an attempt to prevent nitrate tachyphylaxis and nitrate side effects in humans. GTN exerts its potent vasodilator effects by release of an NO-related species. At the same time, GTN causes release of ROS from mitochondria and (upon chronic therapy) multiple other cellular sources (Münzel et al., 2005a). Although accumulation of these ROS upon chronic therapy has a number of side effects, short-term exposure to GTN-derived ROS might have paradoxically protective ones, via induction of a phenotype similar to ischemic preconditioning (Dragoni et al., 2007). In this perspective, an easily accessible parameter that allows monitoring the oxidative changes induced by GTN during chronic therapy would be a clinically valuable tool to prevent nitrate oxidative damage while maintaining the beneficial effects of these drugs.

Likewise, we demonstrate for the first time that oral GTN and PETN treatments at clinically relevant doses, although having comparable vasodilator properties, critically differ in...
terms of ALDH-2 inhibition in humans. Treatment with PETN significantly did not modify ALDH-2 activity, nitrate bioactivation, or serum antioxidative capacity (Fig. 7), which confirms previous findings of an antioxidant effects of this drug (Jurt et al., 2001; Gori et al., 2003; Oberle et al., 2003; Wenzel et al., 2007c). Previous observations suggested induction of cross-tolerance to GTN by repeated doses of PETN (Schelling and Lasagna, 1967). However, our own recent observations from a double-blind, placebo-controlled clinical trial on the vascular effects of chronic PETN therapy in coronary artery disease patients indicate that chronic PETN treatment rather improves NMD (Schiewe et al., 2008). Previously published data demonstrated that NMD is a reliable marker for impaired GTN potency as found in the setting of nitrate tolerance (Hu et al., 2007). Recent findings from our group indicate that the family of nitrates is quite heterogeneous with respect to their bioactivation process and their capacity to stimulate vascular superoxide production along with the development of tolerance and endothelial dysfunction. Although isosorbide dinitrate, isosorbide-5-mononitrate, and GTN result in clinical tolerance after chronic treatment (Schulz et al., 2002; Sekiya et al., 2005; Thomas et al., 2007), treatment of experimental animals and humans with the organic nitrate PETN is devoid of tolerance development and preserves endothelial function (Jurt et al., 2001; Gori et al., 2003). In contrast to all other available organic nitrates, PETN and its metabolites induce the antioxidant defense protein HO-1, thus increasing the formation of the antioxidant molecule bilirubin and the signaling molecule carbon monoxide (Oberle et al., 1999, 2003; Wenzel et al., 2007c). In addition, increased expression of HO-1 in response to PETN treatment also leads to a marked increase in the expression of ferritin via HO-1-dependent release of free iron from endogenous heme sources (Oberle et al., 1999). Finally, we showed recently that in vivo PETN administration, compared with GTN, does not affect the nitrate esterase activity of ALDH-2 and does not elicit ROS formation in isolated arteries and mitochondria. This is probably due to HO-1 up-regulation by PETN, which results in increased levels of the highly efficient peroxynitrite scavenger bilirubin and an increase in the expression of ferritin, subsequently leading to the prevention of vascular oxidative injury. Additional considerations on the role of ALDH-2 for the bioactivation of...
organic nitrates and in vitro versus in vivo nitrate tolerance are presented in supplemental data.

Finally, we demonstrate that chronic GTN treatment induces impaired acetylcholine- and GTN-dependent relaxation (Fig. 5). Although the latter is probably due to an inactivation of the GTN bioactivating enzyme ALDH-2, the impaired endothelial function (ACh response) reflects the induction of oxidative stress and subsequent NO inactivation rather than desensitization of NO/cGMP signaling (by overcharge with endogenous vasodilators) (Schulz et al., 2002). It is noteworthy that a single sublingual administration of GTN in humans not only impairs the GTN bioactivating enzyme ALDH-2 (Figs. 2–4) but also creates a considerable “pool of vasodilators,” such as S-nitrosoproteins and other NOx species in plasma (Fig. 2) and whole blood. These high concentrations of S-nitrosothiols may cause a desensitization of the NO signaling pathway, which may in turn be misinterpreted as endothelial dysfunction because it manifests as an impaired ACh-elicited relaxation (Fig. 4A). The involvement of ROS/RNS formation in this process is a key event for nitrate-induced vascular dysfunction because not only does it inactivate the redox-sensitive ALDH-2, but it also decreases serum antioxidative capacity and, likewise, triggers mitochondrial ROS formation. This desensitization of the NO signaling pathway has been demonstrated previously for repeated nitrovasodilator infusions in rabbits, which resulted in a maximal decrease in blood pressure that was not further augmented by additional administrations of nitrovasodilators (Agvald et al., 1999).

We would like to emphasize that our data are in line with two recent studies using ALDH-2 in white blood cells in another context. 1) Because peripheral blood mononuclear cells (PBMCs) contain large amounts of ALDH-2 protein that is highly active, endothelial progenitor cells as a subfraction of PBMCs can be analyzed by fluorescence-activated cell sorting using the expression of ALDH-2 as a marker (Povsic et al., 2007). 2) ALDH-2 activity in human PBMCs was used to characterize the role of calcitonin gene-related peptide in nitroglycerin-mediated cardiovascular effects (Guo et al., 2008). Beyond the role of ALDH-2 for organic nitrate bioactivation, a very recent publication in Science provided strong evidence that ALDH-2 activation (e.g., by ethanol and protein kinase Cε stimulation) is associated with a reduction of ischemic damage to the heart; vice versa, inhibition of ALDH-2 (e.g., by cyanamide or nitroglycerin) increases infarct size in an experimental model of myocardial infarction (Chen et al., 2008). These authors identified a new phosphorylation site in ALDH-2 involved in regulation of enzymatic activity and an endogenous, physiological activator (Alda-1) determining the cardioprotective action of ALDH-2 in vivo. This report provides a mechanistic basis for previous reports by our group on increased cardiotoxicity in ALDH-2-deficient mice (Wenzel et al., 2008a) and aggravation of mitochondrial oxidative stress and endothelial dysfunction in aged ALDH-2 knockout mice (Wenzel et al., 2008b). In view of this new clinical implication of ALDH-2 in ischemic heart disease, reliable measurement of ALDH-2 activity in WBCs seems to be an attractive marker to assess potential ischemic damage to the heart.

Limitations of the Study. The major limitation of the study may be that not all parameters were measured in the same number of volunteers (e.g., peripheral vascular function, nitrate bioactivating capacity, and thromboxane levels were measured in three to four volunteers only; the number of investigated individuals is clearly stated in the legend to figures). The last appreciable limitation is that part of the study was done in rats because chronic GTN treatment is associated with serious side effects for the volunteers (e.g., headache).

Conclusions and Clinical Implications. In conclusion, our results establish WBC ALDH-2 activity as a valuable marker of human in vivo nitrate tolerance to monitor nitrate-induced vascular side effects. Furthermore, this assay will facilitate the development of new organic nitrates. Although maintaining the hemodynamic effects of GTN, desirable new nitrate compounds will not cause oxidative stress and, therefore, preserve ALDH-2 function and vasodilating action. In the present contribution, we propose the use of PETN (Fig. 7) or of the ALDH-cofactor LA (coadministered with GTN) as feasible and effective strategies to prevent the loss of ALDH-2 activity and the formation of ROS (Fig. 7). Overall, these observations emphasize the importance of the interaction between GTN and the mitochondria as a determinant for nitrate action, side effects, and tolerance development and suggest that these recently acquired notions might have practical clinical implications.

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