when given acutely, organic nitrates such as nitroglycerin (glyceryl trinitrate, GTN) have potent antiischemic effects.1 Their clinical usefulness during long-term treatment, however, is limited because of the development of tolerance and cross-tolerance to endothelium-dependent and independent vasodilators.2 The phenomenon of nitrate tolerance was observed first in response to treatment with GTN,3 but seems also to be shared by other organic nitrates such as isosorbide dinitrate (ISDN)4 and mononitrate (ISMN), but interestingly not by pentaerythrityl tetranitrate (PETN).5,6

**See page 1673**

Recently, a novel bioactivation pathway for GTN was reported, and the mitochondrial aldehyde dehydrogenase (ALDH-2) was identified as the bioactivating enzyme.7 The link between GTN-induced oxidative stress and the development of nitrate tolerance was established by the demonstration of increased reactive oxygen species (ROS) and reactive nitrogen species (RNS) production within mitochondria,8 which inactivate the ALDH-2 by oxidizing sulphydryl (SH)-groups in the active center of the enzyme.9

Further evidence for an important role of mitochondria in the development of nitrate tolerance was provided in studies with mice with partial manganese superoxide dismutase deficiency (Mn-SOD−/− mice). With these studies we could demonstrate that Mn-SOD−/− mice had a significantly higher susceptibility for the development of nitrate tolerance compared with wild-type mice.10 Further support for a causal involvement of mitochondrial ROS in nitrate tolerance came from experiments where mitochondria-targeted antioxidants were able to prevent the development of tolerance.11

Recently, we were able to demonstrate that PETN bioactivation is also mediated by the ALDH-2 and therefore that this organic nitrate shares the same biotransformation process.
like GTN.12,13 When isolated aortic rings were pretreated with high doses of PETN or GTN to induce in vitro tolerance and subsequently challenged with low concentrations of PETN, the loss of vasodilator responsiveness (tolerance) was much more pronounced in GTN-pretreated vessels, indicating that GTN induced a marked degree of cross-tolerance to PETN. This finding is consistent with several animal14,15 and human studies5,6,16 showing that treatment with PETN failed to induce tolerance.

The development of nitrate tolerance has been demonstrated to be associated with increased vascular production of ROS.17 Accordingly, cotreatment with antioxidants such as vitamin C18 as well as folate19 or substances that reduce oxidative stress indirectly such as ACE-inhibitors20 were able to prevent the development of the nitrate tolerance.

In vitro studies with isolated cells have shown that PETN treatment upregulates the expression of heme oxygenase-1 (HO-1) and subsequently ferritin, which have been described as strong antioxidative properties.21,22 Whether this treatment finally results in tolerance remains to be established.

Based on these findings we sought to determine whether or not in vivo treatment with PETN will cause tolerance to PETN and cross-tolerance to GTN and the endothelium-dependent vasodilator acetylcholine (ACh), respectively, and whether a lack of induction of tolerance and endothelial dysfunction may lead to an aggravation or inhibition of tolerance in response to 4-day treatment with GTN or PETN.

Materials and Methods
Male Wistar rats (Charles River, Sulzfeld, Germany) were infused for 4 days with either GTN (6.6 μg/kg/min in ethanol or the solvent as a control) or PETN (10.5 μg/kg/min in DMSO or the solvent as a control). PETN and its metabolites were detected in whole blood using a high-performance liquid chromatography (HPLC)-based method with chemiluminescent nitrogen detection (CLND). Hemodynamic effects (blood pressure) were assessed telemetrically in freely moving animals. Endothelial and smooth muscle function were measured in organ chambers by isometric tension experiments—the vasodilation in response to endothelium-dependent acetylcholine or -independent GTN. Vascular NO/cGMP signaling was determined by phosphorylation of VASP (P-VASP) on Western blot, protein expression by SDS-PAGE followed by Western blotting. Vascular and cardiac mRNA was assessed by real-time polymerase chain reaction (PCR). HO-1 activity was measured by serum bilirubin levels. Vascular and cardiac oxidative stress was determined using L-012 enhanced chemiluminescence. ALDH activity was measured by conversion of a benzaldehyde derivative to its benzoic acid product using an HPLC-based assay. Key experiments consisted of the induction of HO-1 by hemin (1×25 mg/kg, i.p.) and suppression by apigenin (10 mg/kg/d) in GTN- or PETN-treated rats as well as measurement of the above described parameters in these animals. For detailed protocols please the supplemental materials, available online at http://atvb.ahajournals.org.

Results
PETN Metabolites in Whole Blood
The successful administration of PETN was verified by the levels of its metabolites in whole blood. The results are summarized in supplemental Figure I and supplemental Table I.

Hemodynamic Responses
On the first day of therapy, MAP was significantly decreased in both groups (GTN and PETN) (P<0.05). After 6 days of continuous treatment, MAP was still decreased in PETN-treated animals, whereas in the GTN-group MAP was even higher than baseline compatible with hemodynamic tolerance to GTN, but not PETN (Figure 1A).

As shown in Figure 1C through 1E, PETN caused neither tolerance nor cross-tolerance to GTN and ACh. In contrast, GTN in vivo treatment caused tolerance to GTN, cross-tolerance to PETN, and endothelial dysfunction (Figure 1C through 1E and supplemental Table II). One should also note that the trinitrate metabolite of PETN, pentaerythritol trinitrate (PETriN), showed similar vasodilator potency as compared with GTN, but did not induce tolerance or cross-tolerance...
Effect of PETN or GTN In Vivo Treatment on ROS Formation, ALDH, and Heme Oxygenase Activities

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Rats were infused for 4 days with either the solvent (DMSO) alone or PETN in solvent. *Significant difference (P<0.05) vs control.

PETN Treatment Increases and GTN Treatment Decreases the Activity of the cGMP-Dependent Kinase

Phosphorylation of the vasodilator-stimulated phosphoprotein (VASP) at serine 239 reflects cGMP-dependent protein kinase I (cGK-I) activity and accordingly vascular NO bioavailability. P-VASP levels in aortic tissue from PETN-treated animals were significantly higher compared with controls (Figure 1B), whereas GTN treatment lead to a marked decrease in P-VASP levels as before23 (Figure 1B).

Mitochondrial and Vascular Reactive Oxygen and Nitrogen Species Formation

In vivo treatment with PETN did not stimulate ROS (quantified by chemiluminescence) formation in isolated heart mitochondria nor in intact aortic vessel segments (Table). In contrast, as shown before,24 in vivo treatment with GTN significantly increased mitochondrial and vascular ROS formation (Table).

ALDH-2 Dehydrogenase and Esterase Activity

Mitochondrial ALDH-2 and total vascular ALDH dehydrogenase activity, as well as esterase activity, were not modified by in vivo PETN treatment (Table). In contrast, in vivo GTN therapy decreased mitochondrial ALDH-2 activity, vascular ALDH, as well as esterase activity (Table).

Effects of In Vivo Treatment With GTN or PETN on Expression of ALDH-2, HO-1, Endothelial NO Synthase (eNOS), Soluble Guanylyl Cyclase (sGC), and HO-1 Activity

The expression of the GTN and PETN bioactivating enzyme ALDH-2 was significantly decreased in vessels from in vivo GTN-treated rats, but not in vessels from PETN-treated animals (Figure 2A). In addition, cardiac ALDH-2 protein expression was decreased by GTN (supplemental Figure VI). PETN, but not GTN increased HO-1 expression in the aorta (Figure 2A). Cardiac HO-1 protein expression was significantly decreased in GTN-treated rats (supplemental Figure VI). eNOS expression was increased in response to treatment with both organic nitrates (Figure 2B). The expression of the sGC subunit β1 was increased by GTN and not by PETN treatment (Figure 2B). PETN infusion increased plasma (supplemental Figure V). This protective effect of PETN and PETriN was also not mimicked by NO-independent vasodilators of the Ca-antagonist group, amloidipine and nifedipine ( supplemental Figure IX). Interestingly, GTN in vivo treatment induced no cross-tolerance to ISMN but mild cross-tolerance to ISDN (supplemental Table III).

**Figure 2.** Protein expression of ALDH-2 and HO-1 (A) as well as eNOS and sGCβ1 (B) in isolated aortic vessel segments of rats upon treatment with PETN or GTN. A: ALDH-2 expression decreased significantly by GTN but not by PETN treatment. HO-1 expression was not changed in response to GTN, but significantly increased in response to PETN in vivo treatment. B: eNOS expression was increased significantly in response to both organic nitrates whereas sGC expression was upregulated solely in GTN-treated rats. Below the densitometric data the representative original blots are shown. Data are mean±SEM of n=4–6 (ALDH-2), 15–28 (HO-1), 12–16 (eNOS), and 15–23 (sGC) independent experiments. *P<0.05 vs solvent control.
bulirubin levels compatible with increased HO-1 activity. In contrast, GTN treatment decreased bilirubin levels (Table).

HO-1 and Ferritin mRNA Expression
HO-1 mRNA expression was slightly but significantly increased by GTN treatment (supplemental Figure II), whereas in vivo PETN administration markedly upregulated HO-1 mRNA levels in accordance with the protein expression data (supplemental Figure II). A similar observation was made for mRNA levels of ferritin. GTN in vivo increased ferritin expression significantly in the heart, whereas no change was observed in the aorta (supplemental Figure II). In response to PETN treatment, the increase in ferritin expression was markedly stronger (supplemental Figure II).

Induction and Inhibition of HO-1 Protein Expression by Hemin and Apigenin
Single i.p. injection of GTN-treated rats with the HO-1 inducer hemin increased vascular HO-1 mRNA expression and protein (Figure 3C and 3D). Subsequently, tolerance was prevented and the cGK-I activity was increased (Figure 3A and 3B). In addition, oxidative stress within mitochondria was reduced and ALDH-2 activity improved significantly (Figure 3E and 3F). Coinfusion of PETN-treated rats with the inhibitor of HO-1 expression apigenin induced a “tolerance-like” state (supplemental Figure IIIa), indicating that PETN-treated rats developed tolerance in the absence of the protective effects of HO-1. Accordingly, apigenin cotreatment increased mitochondrial ROS formation (supplemental Figure IIIb). The decrease of mitochondrial oxidative stress by HO-1 induction by hemin and the increase of ROS formation induced by HO-1 suppression with apigenin could be attributed at least in part to increased or decreased bilirubin levels. Indeed, we could demonstrate the potent antioxidant properties of bilirubin, by demonstrating a concentration-dependent inhibitory effect on mitochondrial ROS formation in isolated mitochondria from rats treated in vivo with GTN (supplemental Figure IV). Finally, the HO-1 inducer hemin significantly stimulated the expression of eNOS protein in aortas from GTN-treated rats, whereas apigenin, an HO-1 inhibitor, had no effect on eNOS protein levels in PETN-treated rats at all (supplemental Figure VII).

Discussion
The results of the present study demonstrate that 2 different organic nitrates such as GTN and PETN, both being bioactivated by the enzyme ALDH-2, have marked differing effects on the development of tolerance, oxidative
stress, ALDH-2 expression, and desensitization to the endothelium-dependent vasodilator ACh (so-called cross-tolerance). PETN and GTN treatment upregulated eNOS protein while GTN in addition upregulated sGC expression. The vascular levels of phosphorylated VASP, however, indicated that cGK-I activity was increased solely in response to long-term PETN treatment. Upregulation of HO-1 by hemin prevented tolerance to GTN whereas inhibition of HO-1 activity by apigenin induced a “tolerance-like” state in PETN-treated rats, pointing to a crucial role of this enzyme in the modulation of the degree of tolerance in response to the use of organic nitrates. This is the first description of PETN effects on these parameters in rats whereas GTN was used for in-study comparison. The suppression of GTN-induced tolerance by HO-1 upregulation is a new observation.

As we have demonstrated recently, the development of tolerance in response to organic nitrates correlates strongly with their capacity to stimulate mitochondrial ROS/RNS formation and to inhibit ALDH-2 activity.12 Importantly, GTN, PETN, and its metabolite PETn correlate to be bioactivated by ALDH-2, whereas ISDN, ISMN, pentaerithrityl dinitrate (PEDN), and pentaerithrityl mononitrate (PEMN) are likely to be bioactivated by other pathways, such as cytochrome P450 systems.13,25 Interestingly, in contrast to GTN, PETN induces markedly less tolerance “in vitro”, mitochondrial ROS/RNS formation, and inhibition of ALDH-2 activity, when being challenged acutely with high concentrations of this compound,12 suggesting that “antioxidant” properties may come into play.26

Indeed, in contrast to GTN, PETN treatment did not stimulate vascular ROS production (Table) and in addition did not inhibit the activity of the bioactivating enzyme ALDH-2 (Table). Likewise, GTN but not PETN treatment resulted in a significant downregulation of this nitrate reductase (Figure 2A). This explains, as shown before, why treatment with GTN induces cross-tolerance to PETN, whereas PETN treatment does not desensitize the vasculature to GTN.12 These findings also indicate that, in addition to an inhibition of the activity of the ALDH-2, a downregulation of the bioactivating enzyme also contributes to the tolerance phenomenon in response to chronic GTN treatment.

One candidate responsible for the antioxidant properties of PETN is the HO-1. Previous in vitro studies have indicated that incubation of cultured endothelial cells with PETN upregulates HO-1 and subsequently increases intracellular bilirubin, ferritin, and carbon oxide levels, all of which may explain at least in part the antioxidant effects of this organic nitrate.22 To address whether this may occur also in response to in vivo treatment with organic nitrates, we treated Wistar rats with GTN and PETN, respectively. In the present study, male Wistar rats were treated for 4 days with PETN using the infusion method which was adopted from the previously established protocol in our laboratory to induce tolerance in Wistar rats via GTN infusion (6.6 μg/kg/min).3,24 The infusion rate of PETN (10.5 μg/kg/min) was similar to that of GTN.

Treatment of Wistar rats with both organic nitrates resulted in marked differences with respect to their effects on the antioxidant enzyme HO-1. Whereas GTN slightly modified HO-1 expression at the mRNA and protein level, a marked increase was established in response to PETN treatment in the vascular tissue, but also in the heart (Figure 2A and supplemental Figure II). HO-1 is the rate limiting enzyme in heme degradation to generate equimolar amounts of biliverdin, ferrous free iron, and carbon monoxide. Subsequently biliverdin is converted to bilirubin by the biliverdin reductase, and free iron is sequestered by ferritin. Thus, the upregulation of HO-1 was, as expected, paralleled by an increase in the expression of ferritin (supplemental Figure II). Indirect evidence for an upregulation of this antioxidant system was provided by the observed increase in plasma bilirubin levels in response to PETN but not GTN, reflecting increased HO-1 activity (Table).

The data summarized in supplemental Figure IV clearly indicate that bilirubin is a powerful inhibitor of ROS formation in mitochondria of animals treated in vivo with GTN. Bilirubin is more efficient than vitamin E in preventing lipid peroxidation in vitro.27 In addition, higher serum levels are inversely related to the incidence of coronary artery disease.28 Bilirubin prevents the activation of the vascular NADPH oxidase29 and inhibits protein kinase C activity,30 mechanisms that have been proposed to be mechanistically involved in the development of nitrate tolerance.31,32

The induction of ferritin expression has been shown to provide marked antioxidant cellular protection by rapidly sequestering free cytosolic iron, the crucial catalyst of oxygen-centered radical formation via the Fenton reaction in biological systems.33 Furthermore, the increase in expression of intracellular ferritin has been shown to reduce the cytotoxic effects of hydrogen peroxide in vascular endothelial cells.34 Thus, it is conceivable to conclude that, although the metabolism of both PETN and GTN lead to increased ROS/RNS production,12 the consequences for the metabolism of PETN can be neglected because of its simultaneous stimulatory effects on the activity and expression of HO-1, ferritin, and increased bilirubin levels.

Next we studied the effects of treatment with organic nitrates on the expression of the eNOS and the sGC and the activity of the cGK-I. As shown before, GTN treatment upregulated eNOS and sGC expression associated, however, with a reduction of the vascular levels of phosphorylated VASP compatible with an inhibition of cGK-I activity (Figures 2B and 1B). In contrast, PETN treatment upregulated the expression of eNOS, which was paralleled by increase in P-VASP levels, which nicely fits with our observation that in contrast to GTN, PETN treatment beneficially influences NO, sGC, cGK-I signaling and therefore does not induce endothelial dysfunction (Figure 1C and supplemental Table II). However, one should note that PETN-dependent HO-1 induction leads also to increased CO formation, which itself, although it is a weak activator of sGC, could, at least in part, be responsible for the increased P-VASP formation in the PETN treated group.

The proof of concept was provided by experiments using the HO-1 inducer hemin in GTN-treated animals and the
suppressor of HO-1, apigenin, in PETN-treated rats, respectively. Hemin treatment increased HO-1 expression and simultaneously prevented the development of GTN tolerance, reduced mitochondrial ROS formation, and increased mitochondrial ALDH-2 activity (Figure 3). In contrast, the suppressor of HO-1 apigenin was able to significantly reduce vascular responsiveness to PETN and simultaneously increased mitochondrial ROS formation (supplemental Figure III).

We here provide an explanation why in vivo treatment with PETN is devoid of tolerance and cross-tolerance induction in response to prolonged in vivo treatment. In contrast to GTN, PETN does not increase vascular oxidative stress and therefore does not interfere with its bioactivation by ALDH-2 (supplemental Figure X). A likely explanation for this beneficial property of PETN is the induction of the antioxidant enzyme HO-1 and subsequent increases in the expression of ferritin in vascular tissue but also in the heart. This favorable characteristic of PETN may also explain why therapy with GTN but not PETN causes tolerance and stimulates ROS production in human subjects.5

Future Implications

The exciting new observation is the capacity of inducers or inhibitors of HO-1 to modulate the development of tolerance and endothelial dysfunction in response to organic nitrates. The powerful inhibitory effects, eg, of the HO-1 inducer hemin on the development of tolerance in response to GTN may help to develop new agents, allowing to treat patients with GTN chronically without side effects like tolerance and endothelial dysfunction. The pharmacological profile of PETN, increasing vascular NO and decreasing superoxide levels, markedly resembles that of statins and ACE inhibitors.35 It remains to be established, however, whether PETN may indeed represent the first organic nitrate, which will be able to beneficially influence prognosis in patients with coronary artery disease.

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Disclosures

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References


Heme Oxygenase-1: A Novel Key Player in the Development of Tolerance in Response to Organic Nitrates
Philip Wenzel, Matthias Oelze, Meike Coldewey, Marcus Hortmann, Andreas Seeling, Ulrich Hink, Hanke Mollnau, Dirk Stalleicken, Henry Weiner, Jochen Lehmann, Huige Li, Ulrich Förstermann, Thomas Münzel and Andreas Daiber

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Figure I. HPLC chromatograms of PETN metabolites in whole blood from rats upon chronic treatment with PETN (infusion). The PETN metabolites PETriN and PEDN were measured by HPLC analysis with CLND (nitrogen specific) detection. (A) shows a typical chromatogram of standards (PETN, PETriN and PEDN, each 0.5µM) and (B) a typical chromatogram of a purified blood samples from PETN infused rats.
Figure II. mRNA expression of HO-1 and ferritin (heavy chain) in aorta (A) and heart (B) in response to chronic treatment with PETN or GTN. (A) HO-1 mRNA was moderately increased in aorta from *in vivo* GTN-treated rats whereas it was markedly increased in vessels from chronically PETN-treated animals. A similar observation was made for ferritin mRNA levels. (B) HO-1 and ferritin mRNA levels were significantly increased in heart tissue upon chronic treatment with both organic nitrates whereas the increase was more pronounced in response to PETN treatment. Data are mean±SEM of n=6-10 (aortic ferritin and HO-1) and 6-12 (heart ferritin and HO-1) independent experiments. * P<0.05 vs. solvent control.
Figure III. Inhibition of HO-1 expression by apigenin induces tolerance in PETN-treated rats. Apigenin (10mg/kg/d) was co-infused over 4d together with PETN. Apigenin co-treatment decreased PETN vasodilator potency and induced a tolerance-like right-shift in the PETN concentration-relaxation-curve (A). This observation was accompanied by increased mitochondrial ROS formation (B) and decreased cGK-I activity (P-VASP levels) (C) as well as decreased vascular HO-1 protein expression (D). Data are mean±SEM of n=9-11 (A), 28-43 (B), 4 (C) and 4 (D) independent experiments. In panel C and D two original blots are shown for each group. * P<0.05 vs. PETN treatment.
Figure IV. Effect of bolus bilirubin on ROS formation in isolated heart mitochondria from GTN in vivo treated rats. Mitochondrial ROS levels were determined by L-012 (100µM) ECL in the presence of 2.5mM succinate and bilirubin (0-25µM). Data are mean±SEM of n=4-6 independent experiments. * P<0.05 vs. GTN treatment.
**Figure V.** PEtriN *in vivo* treatment (1µl/h 450mM PETriN in DMSO for 4d) of Wistar rats did neither induce tolerance to PETriN nor cross-tolerance to ACh or GTN (concentration-relaxation-curves from 6-14 independent experiments). PEtriN *in vivo* treatment increased the expression of eNOS (179±32% of control), ALDH-2 (114±10% of control) and HO-1 (154±15% of control), slightly increased P-VASP levels (118±12% of control) but did not alter sGC β1 expression (102±6% of control). Western blot data were obtained from tissue from at least 3 animals per group. In addition, PEtriN *in vivo* treatment decreased mitochondrial ROS formation by 13.4±1.5%. Data were measured in aorta and heart mitochondria from 3 animals per group.
Figure VI. Effect of chronic GTN treatment of rats on cardiac ALDH-2 and HO-1 expression. Hearts were homogenized and tested for proteins using specific antibodies for ALDH-2 and HO-1. GTN treatment caused significantly decreased expression of ALDH-2 and HO-1. Data are mean±SEM of n=4 independent experiments. In the lower panel, two original blots are shown for each protein with samples from 4 different animals in each group. * P<0.05 vs. control.
**Figure VII.** Effect of co-treatment with the HO-1 inducer hemin or the HO-1 suppressor apigenin in chronically GTN- or PETN-infused rats on aortic eNOS expression. Aorta were homogenized and tested for eNOS using a specific antibody. Hemin significantly increased eNOS expression whereas apigenin had no effect. Data are mean±SEM of n=5-6 independent experiments. In the lower panel, two original blots are shown with samples from 2 different animals in each group. * P<0.05 vs. GTN-treated group.

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**Figure VIII.** Two representative original tracings (printouts) for ROS detection in aortic rings from sham (ethanol, EtOH, upper panel) and nitroglycerin (NTG, lower panel) in vivo treated rats. The CL signal was accumulated over 60 s. The last value was used for further analysis. Note: The counts were not yet normalized on dry weight of aortic rings.
### Supplement for Wenzel et al. – Heme oxygenase-1 and nitrate tolerance

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Figure IX. Effect of Ca-Antagonists on GTN-induced tolerance in rats. Amlodipine and nifedipine (3mg/kg/d) was co-infused over 4d (or applied at 10 mg/kg/d in the drinking water) together with GTN using osmotic minipumps (1µl/h containing 40mg/ml solutions in DMSO). Ca-antagonist co-infusion did not improve GTN-induced tolerance (A) and did not suppress GTN-dependent vascular oxidative stress (B). Neither co-infusion with Ca-antagonists nor oral treatment increased vascular or cardiac HO-1 mRNA (C). Data are mean±SEM of experiments performed with at least 4 animals/group.
Figure X. Scheme illustrating the mechanisms underlying the oxidative stress concept of nitrate tolerance in response to GTN treatment and the hypothesized mechanisms underlying the beneficial vascular effects in response to PETN. PETN and GTN are bioactivated by mitochondrial ALDH (ALDH-2) yielding 1,2-glyceryl dinitrate and PETriN respectively as well as a yet undefined nitrogen species (NOx, probably nitrite) that undergoes further reduction by the mitochondrial respiratory chain or acidic disproportionation to form an activator of sGC (probably nitric oxide). GTN treatment induces mitochondrial reactive oxygen and nitrogen species formation (ROS/RNS). These ROS/RNS in turn inhibit the GTN bioactivation process by inactivating ALDH-2 or by inhibiting the repair system of the ALDH-2, which includes lipoic acid as well as a reductase system depending on the NADH or NADPH (lipoic acid reductase (LAR), thioredoxin/thioredoxin reductase (Trx/TrxR) or glutathione/glutathione reductase (GSH/GR)). In contrast to GTN, PETN provides potent anti-oxidative effects by inducing HO-1 and ferritin, which in turn decrease ROS levels and therefore protect the ALDH-2-from ROS mediated inactivation.
**TABLE I. Whole blood levels of PETN and its metabolites upon chronic administration by infusion.**

<table>
<thead>
<tr>
<th>Sample</th>
<th>PETN</th>
<th>PETriN</th>
<th>PEDN</th>
</tr>
</thead>
<tbody>
<tr>
<td>PETN infusion (blood)</td>
<td>n.d.</td>
<td>[3-9] 8-12</td>
<td>[4-11] 7-17 (n=12)</td>
</tr>
</tbody>
</table>

*S.E.M. and mean values are not calculated, because the concentrations in the blood samples were mostly at the LOQ (ranges between LOD (limit of detection) and LOQ (limit of quantification) are included in square brackets.).

**PETN metabolites in whole blood.** The successful administration of PETN was verified by the levels of its metabolites in whole blood samples because the uptake of PETN was reported to differ markedly from other organic nitrates¹,². Importantly, we could detect plasma levels that were similar to those observed in patients during clinical therapy¹,². The mononitrate (PEMN) could not be detected by the employed HPLC protocol due to its short retention time and co-elution with other nitrogen-containing compounds. The dinitrate (PEDN) had a typical retention time of 9.9min and its concentration in whole blood was mostly found to be within the limit of detection (LOD) and the limit of quantification (LOQ) in the range 4–11ng/ml. Occasionally, higher concentrations in the range of 17–33ng/ml were determined irrespective of the used administration protocol. The active metabolite trinitrate (PETriN) was detected in blood samples at 18.3min and could be quantified above the LOQ (9ng/ml) to be 12.3ng/ml.
after infusion of PETN. PETN (eluted at 30.7min) was not detected in every blood sample but could be quantified in the faeces samples in increasing concentrations during treatment.

**TABLE II.** Potency of ACh-, PETN- and GTN- induced relaxation in isolated aortic segments from sham-treated, PETN- or GTN-infused rats with or without HO-1 inducing or suppressing co-treatment (hemin or apigenin).

<table>
<thead>
<tr>
<th>In Vivo Treatment</th>
<th>ACh</th>
<th>PETN</th>
<th>GTN</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO a</td>
<td>7.29±0.06 (n=19)</td>
<td>7.56±0.08 (n=15)</td>
<td>7.52±0.06 (n=8)</td>
</tr>
<tr>
<td>PETN in DMSO a</td>
<td>7.26±0.06 (n=20)</td>
<td>7.53±0.07 (n=16)</td>
<td>7.54±0.08 (n=8)</td>
</tr>
<tr>
<td>PETN/Apigenin a</td>
<td>n.d.</td>
<td>6.45±0.12 (n=9) #</td>
<td>n.d.</td>
</tr>
<tr>
<td>EtOH b</td>
<td>7.36±0.05 (n=22)</td>
<td>7.72±0.04 (n=12)</td>
<td>7.52±0.08 (n=17)</td>
</tr>
<tr>
<td>GTN in EtOH b</td>
<td>7.56±0.07 (n=23)</td>
<td>6.72±0.05 (n=8) *</td>
<td>6.32±0.12 (n=12) *</td>
</tr>
<tr>
<td>GTN/Hemin</td>
<td>n.d.</td>
<td>n.d.</td>
<td>7.28±0.10 (n=8) †</td>
</tr>
</tbody>
</table>

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a No statistical significant differences between PETN and DMSO solvent group were observed. # p<0.05 vs. PETN/DMSO infusion.

b * p<0.05 vs. EtOH infusion. † p<0.05 vs. GTN/EtOH infusion.
TABLE III. Potency of ISMN- and ISDN-induced relaxation in isolated aortic segments from sham-treated or GTN-infused rats. Does GTN in vivo induce cross-tolerance to ISMN and ISDN?

Potency, EC$_{50}$ (-log M)

<table>
<thead>
<tr>
<th>In Vivo Treatment</th>
<th>ISMN</th>
<th>ISDN</th>
</tr>
</thead>
<tbody>
<tr>
<td>EtOH$^a$</td>
<td>2.74±0.17 (n=16)</td>
<td>5.13±0.08 (n=16)</td>
</tr>
<tr>
<td>GTN in EtOH$^a$</td>
<td>2.72±0.15 (n=16)</td>
<td>4.61±0.11 (n=16) $^*$</td>
</tr>
</tbody>
</table>

$^a$ * p<0.05 vs. EtOH infusion.
Material and Methods

Materials

PETN (with 80% (w/w) lactose) was from Fluka (Buchs, Switzerland). GTN (Nitrolingual infusion solution (1mg/ml) or an ethanolic stock (100g/l)) was purchased from G. Pohl-Boskamp (Hohenlockstedt, Germany) and Unikem (Copenhagen, Denmark). L-012 (8-amino-5-chloro-7-phenylpyrido[3,4-d]pyridazine-1,4-(2H,3H)dione sodium salt) was from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals were of analytical grade and were obtained from Sigma-Aldrich, Fluka or Merck.

Animals and in vivo treatment

Male Wistar rats (200g), (Charles River, Sulzfeld, Germany) were equipped with micro osmotic pumps 1003D from Alzet (Cupertino, CA) containing 450mM PETN dissolved in DMSO or the solvent and infusion was maintained for three days at 1µl/h (10.5µg/kg/min). For direct comparison, rats were infused with 450mM GTN (6.6µg/kg/min in ethanol or the solvent as a control). GTN-infusion was performed as previously described ³. All animal treatment was in accordance with the Declaration of Helsinki and with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health and was granted by the Ethics Committee of the University Hospital Eppendorf and the University Hospital Mainz.

Quantification of PETN metabolites in rat whole blood and faeces

The successful administration of PETN and PETriN was controlled by measuring levels of PETN and its metabolites (the trinitrate PETriN and the dinitrate PEDN) in whole blood and faeces samples on day 3 by an HPLC-based method with chemiluminescent nitrogen detection (CLND) as described ⁴.
Measurement of bilirubin

Heme oxygenase activity was determined by measuring serum levels of bilirubin photometrically by a Hitachi 917 R Analyzer (Boehringer Mannheim, Germany) in the Department of Clinical Chemistry, University Hospital, Mainz, according to a standard procedure (2,5-dichlorophenyl diazonium (DPD) method).

Telemetric Measurement of Blood Pressure

Male Wistar rats (from Charles River Breeding Laboratories, Sulzfeld, Germany) were housed in single cages under controlled environmental conditions (23±1°C, light:dark, 12:12h, light on at 7:00 a.m.). Food (regular rat chow) and water were always available. Every cage was positioned on top of a receiver platform. Systolic, diastolic and resulting mean arterial pressures (MAP) were measured telemetrically in freely moving animals by using implanted transmitters as described; an activity index with arbitrary units was calculated by the movement of the rat relatively to the center of the receiver. Measurements were taken every 15min for 10sec in each animal using the DataQuest System (Datasciences, St. Paul). Briefly, measurement catheters were implanted intraabdominally with the telemetric sender sutured to the internal peritoneal layer. The tip of the catheter was inserted into the distal thoracic aorta and glued to the aortic bed. Surgery was carried out under isoflurane (5% inhalant in room air) and ketamine anesthesia. After surgery, rats were allowed to recover for two weeks. To collect baseline blood pressure data, telemetric measurement was initiated in the chronically instrumented rats 12h before implantation of the osmotic minipumps delivering NTG (450mM, 1µl/h/7d) or PETN (450mM, 1µl/h/7d). Telemetry was continued throughout the nitrate treatment. After one week of monitoring, we evaluated the delta in MAP 1 day and 6 days after begin of nitrate therapy. To obtain an average value, we calculated the mean of the MAP values obtained during the resting period of the animal (i.e, approx. 12h during daytime, correlated to the activity index) und substracted it from the average baseline value of MAP.
Isometric tension studies

Vascular reactivities were studied in organ chambers as described. Upon pre-constriction with phenylephrine to 70% of maximal KCl-induced contraction, vasodilators (acetylcholine, PETN, PETriN or GTN) were applied cumulatively and concentration-response curves were recorded using PowerLab hard- and software (ADInstruments Ltd., Spechbach, Germany).

Western Blot analysis

Isolated aortic tissue was frozen and homogenized in liquid nitrogen. Proteins were separated by SDS-Page and blotted onto a nitrocellulose membrane. After blocking, immunoblotting was performed with an antibody against α-Aktinin, heme oxygenase-1 (1:5000, monoclonal, Stressgen, San Diego, CA), P-VASP Ser-239 (1μg/ml, monoclonal, Calbiochem, Darmstadt, Germany), ALDH-2 (1:2000, polyclonal as previously characterized), eNOS (1:1000, monoclonal, Transduction Labs, Lexington, KY) and sGC β1 (1:2000, polyclonal, Calbiochem, San Diego, CA). Detection was performed by ECL with peroxidase conjugated anti–rabbit/mouse secondary antibodies (1:10000, Vector Lab., Burlingame, CA). The antibody-specific bands were quantified by densitometry. The details of the protocol were previously published.

Quantification of mRNA by reverse transcription real-time PCR (qRT-PCR)

mRNA expression of HO-1 and ferritin (heavy-chain) was analyzed with quantitative real-time RT-PCR using an iCycler™ iQ system (Bio-Rad Laboratories, Munich, Germany). Briefly, total RNA from rat aorta and heart was isolated according to the manufacturer's protocol (RNeasy Fibrous Tissue Mini Kit; Qiagen, Hilden, Germany). 0.5μg of total RNA was used for real-time RT-PCR analysis with the QuantiTect™ Probe RT-PCR kit (Qiagen). TaqMan® Gene Expression assays (Applied Biosystems, Foster City, CA) for HO-1, ferritin
and GAPDH were purchased as probe and primer sets. The comparative Ct method was used for relative mRNA quantification. Gene expression was normalized to the endogenous control, GAPDH mRNA, and the amount of target gene mRNA expression in each sample was expressed relative to that of control.

**Detection of mitochondrial and vascular oxidative stress**

Isolated mitochondria were prepared from rat hearts according to a previously published protocol and ROS formation was detected by L-012 (100 µM) ECL as recently described. Briefly, hearts were homogenized and pellets from 1.500g and 2.000g centrifugation were discarded. The 20.000g pellet was resuspended and used for measurements. For detection of vascular oxidative stress thoracic aorta were freshly obtained from the rats, cut into segments of 0.5 cm length and kept in Krebs-Hepes (KH) buffer until use. The segment was equilibrated for 20 min in KH-buffer at 37 °C and then placed in the chemiluminometer, a single photon counter (Lumat, Berthold Techn., Bad Wildbad, Germany) in phosphate-buffered saline (PBS) containing the luminol analogue L-012 (100 µM). The chemiluminescence was detected in 60 s intervals for 20 min and the last value expressed as counts/min. This procedure was adapted from previous studies.

**Mitochondrial ALDH-2 dehydrogenase and vascular dehydrogenase ALDH activity**

The dehydrogenase activity of ALDH-2 in isolated rat heart mitochondria was determined by measuring the conversion of benzaldehyde (200µM) to benzoic acid by a previously published HPLC protocol. ALDH-2 esterase activity in vascular tissue was measured after adding methylbenzoate (1mM) as described. For determination of vascular ALDH dehydrogenase activity, isolated aortic rings were incubated with benzaldehyde and benzoic acid was measured in the incubation solution as described.
**Induction and inhibition of HO-1 protein expression by hemin and apigenin**

To evaluate the influence of HO-1 in the pathophysiology of nitrate tolerance, we co-infused PETN treated rats for 4 days with a down-regulator of HO-1, apigenin (10mg/kg/d) or solvent (DMSO), using osmotic minipumps as described above. Apigenin was recently described as a powerful inhibitor of hemin-induced HO-1 expression \(^{17}\). Likewise, GTN infused rats were given an inducer of HO-1, hemin (25mg/kg, 1mg of hemin dissolved in 20µl DMSO) intra-peritoneally 12h before sacrifice or solvent. Hemin was previously described as a powerful inducer of HO-1 expression \(^{17-19}\).

**Statistical Analysis**

Results are expressed as mean±SEM. If not indicated differently, the n-values indicate duplicate measurements from animals/group, but 4 animals/group were used at minimum. One-way ANOVA (with Bonferroni’s or Dunn’s correction for comparison of multiple means) was used for comparisons of vasodilator potency and efficacy, L-012-derived chemiluminescence, ALDH-2 dehydrogenase and heme oxygenase-1 activity, effects of hemin/apigenin as well as protein and mRNA expression. The EC\(_{50}\) value for each experiment was obtained by log-transformation. P values <0.05 were considered significant. * indicates significance vs. solvent control.
References


