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## **Pentaerythritol Tetranitrate Improves Angiotensin II Induced Vascular Dysfunction via Induction of Heme Oxygenase-1**

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# Pentaerythritol Tetranitrate Improves Angiotensin II–Induced Vascular Dysfunction via Induction of Heme Oxygenase-1

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**Abstract**—The organic nitrate pentaerythritol tetranitrate is devoid of nitrate tolerance, which has been attributed to the induction of the antioxidant enzyme heme oxygenase (HO)-1. With the present study, we tested whether chronic treatment with pentaerythritol tetranitrate can improve angiotensin II–induced vascular oxidative stress and dysfunction. In contrast to isosorbide-5 mononitrate (75 mg/kg per day for 7 days), treatment with pentaerythritol tetranitrate (15 mg/kg per day for 7 days) improved the impaired endothelial and smooth muscle function and normalized vascular and cardiac reactive oxygen species production (mitochondria, NADPH oxidase activity, and uncoupled endothelial NO synthase), as assessed by dihydroethidine staining, lucigenin-enhanced chemiluminescence, and quantification of dihydroethidine oxidation products in angiotensin II (1 mg/kg per day for 7 days)–treated rats. The antioxidant features of pentaerythritol tetranitrate were recapitulated in spontaneously hypertensive rats. In addition to an increase in HO-1 protein expression, pentaerythritol tetranitrate but not isosorbide-5 mononitrate normalized vascular reactive oxygen species formation and augmented aortic protein levels of the tetrahydrobiopterin-synthesizing enzymes GTP-cyclohydrolase I and dihydrofolate reductase in angiotensin II–treated rats, thereby preventing endothelial NO synthase uncoupling. Haploinsufficiency of HO-1 completely abolished the beneficial effects of pentaerythritol tetranitrate in angiotensin II–treated mice, whereas HO-1 induction by hemin (25 mg/kg) mimicked the effect of pentaerythritol tetranitrate. Improvement of vascular function in this particular model of arterial hypertension by pentaerythritol tetranitrate largely depends on the induction of the antioxidant enzyme HO-1 and identifies pentaerythritol tetranitrate, in contrast to isosorbide-5 mononitrate, as an organic nitrate able to improve rather than to worsen endothelial function. (*Hypertension*. 2010;55:897-904.)

**Key Words:** pentaerythritol tetranitrate ■ isosorbide-5-mononitrate ■ angiotensin-II ■ SHR  
■ endothelial dysfunction ■ vascular oxidative stress

Both arterial hypertension and coronary artery disease are associated with an activation of the circulating and local renin-angiotensin system and increased oxidative stress within the vascular wall.<sup>1,2</sup> Angiotensin-II (AT-II) treatment has been shown to cause endothelial dysfunction, which is at least in part mediated by increased vascular reactive oxygen species (ROS) levels.<sup>3,4</sup> ROS sources involved may include the NADPH oxidases,<sup>3</sup> an uncoupled endothelial NO synthase (NOS; eNOS),<sup>4</sup> and mitochondrial superoxide sources.<sup>5</sup> The crucial role of the NADPH oxidase as an important superoxide source was further substantiated by the demonstration that NADPH oxidase 1 overexpression in transgenic mice potentiates AT-II–induced hypertension,<sup>6</sup> whereas blood pressure responses to AT-II were reduced in

NADPH oxidase 1–deficient mice.<sup>7</sup> Increased vascular ROS production and endothelial dysfunction are also accompanied by increased eNOS expression but decreased vascular NO production.<sup>8</sup> Recently, we were able to demonstrate that pharmacological intervention with a statin or an AT-II type 1 receptor blocker improved vascular dysfunction and reduced oxidative stress in an experimental model of diabetes mellitus<sup>9,10</sup> and identified the downregulation of tetrahydrobiopterin (BH<sub>4</sub>) synthesizing enzymes GTP-cyclohydrolase-I (GCH-I) and dihydrofolate reductase (DHFR) as key events for the development of endothelial dysfunction.<sup>8</sup>

Organic nitrates act as endothelium-independent vasodilators of coronary arteries, venous capacity vessels, and collaterals. Nitroglycerin (GTN) is one of the most widely used

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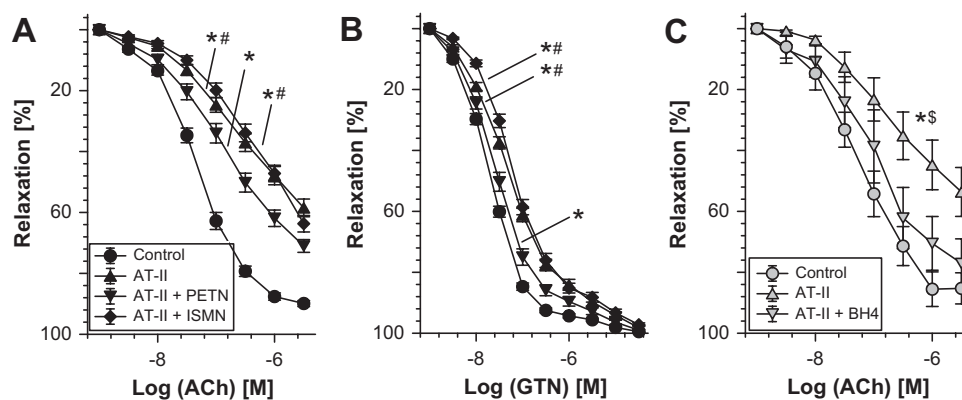
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**Figure 1.** Effects of in vivo PETN (15 mg/kg per day) and ISMN (75 mg/kg per day) treatment on the concentration response relationship to ACh (A) and GTN (B) in aortic rings from AT-II (1 mg/kg per day for 7 days) rats. C, The effects of sepiapterin (100  $\mu$ mol/L), a BH<sub>4</sub> precursor, and polyethylene glycolated-superoxide dismutase (100 U/mL) pretreatment of aortic rings from AT-II-infused rats for 1 hour were determined in separate experiments. Data are the mean  $\pm$  SEM of n=36 to 57 aorta from 10 to 15 rats per group (A and B) and n=6 to 8 from 3 rats per group (C).  $P < 0.05$  vs \*control/DMSO; vs #AT-II+PETN; vs \$AT-II/BH<sub>4</sub>. The statistics were on the basis of 1-way ANOVA comparison of pD<sub>2</sub> values and efficacies (see Table S1) but also on comparisons of all concentrations in all groups by 2-way ANOVA (for sake of clarity, significance is not shown for all of the data points).

anti-ischemic drugs for more than a century. The chronic efficacy of nitrates, however, is blunted because of adverse effects, such as the development of nitrate tolerance and endothelial dysfunction. Recent data indicate that GTN-induced ROS formation accounts for both phenomena, as ROS formed in response to GTN therapy because both phenomena can be corrected by treatment with antioxidants.<sup>11,12</sup> Treatment with mononitrates and dinitrates also causes nitrate tolerance and endothelial dysfunction,<sup>13</sup> although both compounds are clearly not bioactivated by the mitochondrial aldehyde dehydrogenase 2.<sup>14</sup> These findings may explain results from a retrospective analysis indicating increased mortality in response to treatment of patients with myocardial infarction with mononitrates and dinitrates.<sup>15</sup> Among all of the organic nitrates, the most frequently used compounds in the treatment of coronary artery disease are composed of GTN, pentaerythritol tetranitrate (PETN; in Germany), isosorbide dinitrate, and isosorbide-5 mononitrate (ISMN; United States).

More recently, we and others were able to demonstrate that different organic nitrates have distinct pharmacological effects with respect to their bioactivating process and their capacity to stimulate vascular superoxide production. For instance, we demonstrated that the nitrate PETN, while still undergoing mitochondrial activation by the aldehyde dehydrogenase 2,<sup>12</sup> does not induce tolerance or stimulate vascular superoxide production.<sup>12</sup> Although GTN caused a strong oxidative stress-mediated inhibition of the mitochondrial aldehyde dehydrogenase 2, PETN induced an upregulation of the antioxidant enzyme heme oxygenase (HO)-1, which preserved aldehyde dehydrogenase 2 activity. These observations from experimental animal studies were substantiated by clinical data obtained in humans indicating that, in contrast to GTN, PETN does not induce tolerance<sup>16</sup> or endothelial dysfunction.<sup>17</sup>

On the basis of these considerations, we investigated in the AT-II infusion model and in spontaneously hypertensive rats (SHR) to what extent different organic nitrates, such as PETN and ISMN, are able to modulate oxidative stress, endothelial dysfunction, and tolerance development.

## Methods and Materials

### Materials

PETN was obtained from Actavis. GTN was used from a Nitrolin-gual infusion solution (Pohl-Boskamp). All of the other chemicals were purchased from Sigma-Aldrich, Merck, or Fluka.

### Animal Models, In Vivo Infusion of AT-II, and Spontaneously Hypertensive Rats

All of the animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the National Institutes of Health and were granted by the University Hospital Mainz Ethics Committee. Male Wistar rats (250 g) were treated with either AT-II (1.0 mg/kg per day) or solvent (0.9% NaCl) for 7 days, as described previously.<sup>18</sup> Male spontaneously hypertensive rats (SHRs) and Wistar-Kyoto control rats were obtained from Charles River Laboratories (Sulzfeld, Germany). Animals from both groups were treated with either PETN (15 mg/kg per day) or ISMN (75 mg/kg per day) versus vehicle (dimethyl sulfoxide [DMSO]). Male HO-1<sup>+/+</sup> or HO-1<sup>+/-</sup> mice on a 129sv $\times$ BALB/c background were treated with PETN (75 mg/kg per day; DMSO, 0.5  $\mu$ L/h) for 7 days,<sup>19</sup> high-dose AT-II (1.0 mg/kg per day) versus solvent (0.9% NaCl) for 7 days with and without the HO-1 inducer hemin (25 mg/kg single IP injection, 12 hours before being euthanized), or low-dose AT-II (0.1 mg/kg per day) versus solvent (0.9% NaCl) with or without PETN (75 mg/kg per day) for 7 days. For details see the Extended Methods section in the online Data Supplement, available at <http://hyper.ahajournals.org>.

### Vascular Reactivity Studies

Vasodilator responses to the endothelium-dependent vasodilator acetylcholine (ACh) and the endothelium-independent vasodilator GTN were determined in organ chambers by isometric tension studies using phenylephrine-precontracted aortic ring segments, as described previously.<sup>18</sup> Murine aorta were precontracted by prostaglandin F<sub>2 $\alpha$</sub>  as published previously.<sup>19,20</sup>

### Assessment of Vascular and Cardiac Oxidative Stress

Vascular and cardiac oxidative stress were assessed by L-012 (a luminol derivative) or lucigenin-enhanced chemiluminescence (ECL) and dihydroethidine (DHE)-dependent fluorescence, as described elsewhere.<sup>10,14,18,21</sup> For details see the Extended Methods section in the online Data Supplement. For determination of cardiac ROS in mice, 2 to 3 hearts were pooled and mitochondria and membrane fractions were isolated as published recently.<sup>11,19</sup>

### Western Blot Analysis and RT-PCR

Western blotting against eNOS, GCH-1, DHFR, and HO-1 was performed as described previously.<sup>10,21</sup> 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). TaqMan Gene Expression assays (Applied Biosystems) for HO-1 and GAPDH were purchased as probe and primer sets, and gene expression was normalized to the endogenous control GAPDH mRNA as described.<sup>21</sup> For details see the Extended Methods section in the online Data Supplement.

### Statistical Analysis

Results are expressed as mean  $\pm$  SEM.  $pD_2$  values (potencies) were obtained by logit transformation. One-way ANOVA (with Bonferroni or Dunn correction for comparison of multiple means) was used for comparisons of vasodilator potency and efficacy and vascular and cardiac ROS formation and aortic protein and mRNA expression.  $P$  values  $<0.05$  were considered significant.

## Results

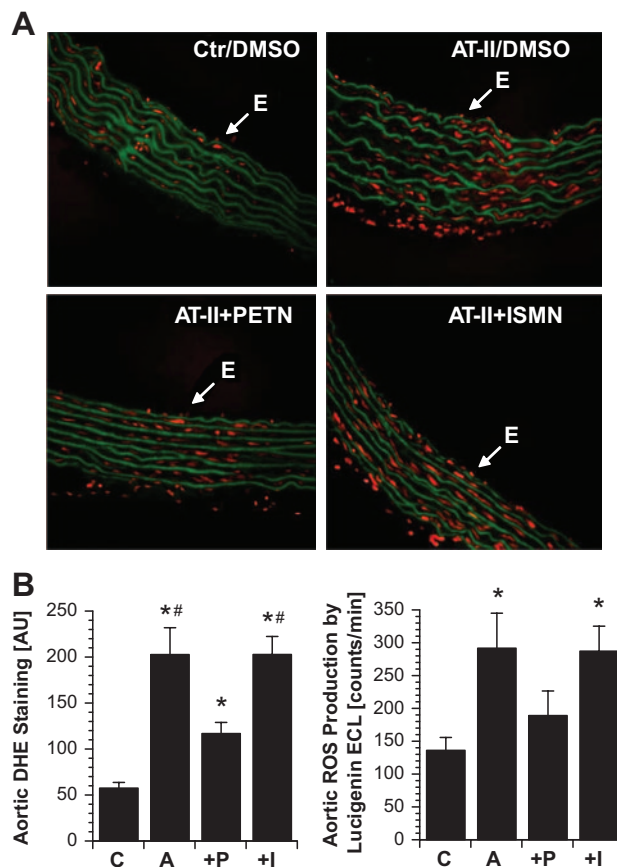
### Effects of PETN and ISMN Cotherapy on AT-II-Induced Vascular Dysfunction

Weight gain in solvent-treated control animals was  $50 \pm 6$  g in 1 week, whereas AT-II treatment caused weight loss of  $61 \pm 16$  g. Weight loss in AT-II-treated animals was significantly improved in hypertensive rats by PETN cotherapy ( $3 \pm 17$  g), whereas ISMN treatment had no significant effect ( $-13 \pm 18$  g). In vivo treatment with PETN rather improved endothelial dysfunction in AT-II-treated animals (Figure 1A and Table S1 in the online Data Supplement), whereas ISMN further impaired AT-II-induced endothelial dysfunction. AT-II infusion-induced cross-tolerance to GTN was corrected by PETN cotreatment but not by ISMN (Figure 1B and Table S1). Treatment of vessels from AT-II-infused rats with the  $BH_4$  precursor sepiapterin normalized endothelial function (Figure 1C). Sensitivity of aorta to vasoconstriction by KCl and phenylephrine was not changed (Table S2). Blood pressure data are presented in Figure S1 and show that AT-II-infused rats and SHR are valid models of experimental hypertension. The beneficial effects of PETN and sepiapterin on vascular function were almost absent in SHR, but ISMN further impaired this parameter (Figure S2).

### Effects of PETN and ISMN Cotreatment on Vascular and Cardiac ROS Production, as Well as eNOS Uncoupling in AT-II-Induced Hypertension

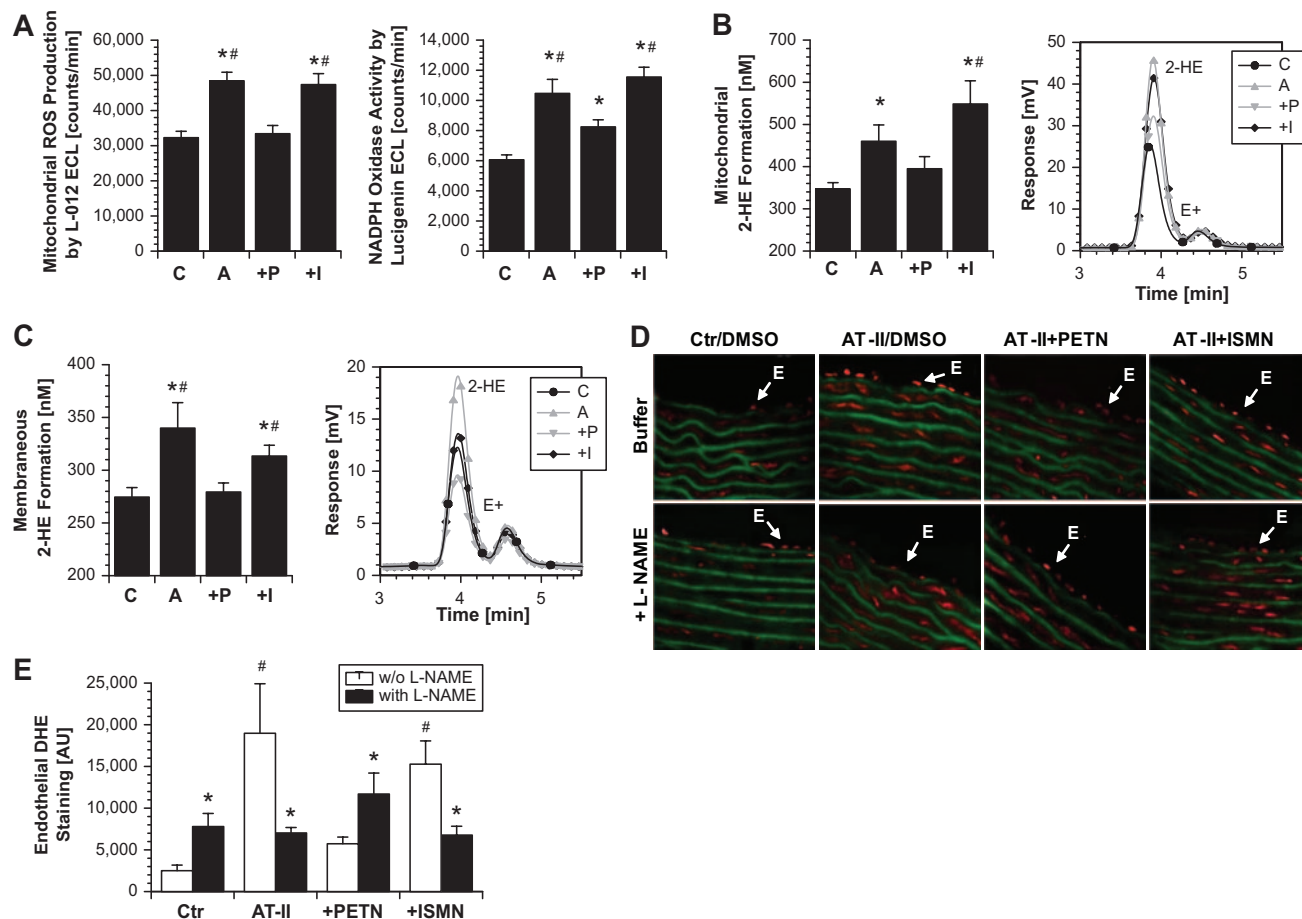
DHE staining (fluorescent microtopography) demonstrated increased vascular superoxide throughout the vessel wall and the endothelium in vessel cryosections from AT-II-treated animals compared with controls (Figure 2A and 2B). Although vascular superoxide in hypertensive rats was not modified by ISMN treatment, a marked reduction was observed under PETN therapy (Figure 2A and 2B). Lucigenin ECL in intact aortic ring segments yielded qualitatively similar results (Figure 2B). AT-II-stimulated mitochondrial ROS formation and NADPH oxidase activity in tissue from the heart were normalized by PETN but not by ISMN treatment (Figure 3A through 3C).

To assess the contribution of an uncoupled eNOS to superoxide formation because of eNOS uncoupling, rat aortic tissue was incubated with an NOS inhibitor,  $N^G$ -nitro-L-



**Figure 2.** Effects of in vivo PETN and ISMN treatment on vascular superoxide levels (A and B) in AT-II rats. A, Transverse aortic cryosections were labeled with DHE ( $1 \mu\text{mol/L}$ ), which produces red fluorescence when oxidized by ROS. Lamina autofluorescence is green. Pictures shown are representative for  $\geq 6$  animals per group. E indicates endothelium. B, Densitometric quantification of the DHE-derived ROS signal throughout the vessel wall (left) and lucigenin ( $5 \mu\text{mol/L}$ )-ECL in intact aortic ring segments (right). The data are mean  $\pm$  SEM of  $n=18$  to 19 experiments with tissue from  $\geq 10$  animals per group.  $P < 0.05$ : vs \*control/DMSO; vs #AT-II+PETN. C indicates control; A, AT-II treated; P, AT-II and PETN treated; I, AT-II and ISMN treated.

arginine methylester (L-NAME). L-NAME increased DHE-derived fluorescence within the endothelial monolayer from control rats (marked with “E” in Figure 3D), whereas the signal in the media was not changed. Inhibition of NOS in control aorta eliminates basal NO production, leading to higher superoxide steady-state levels (which is otherwise scavenged by NO). In contrast, NOS inhibition in vessels from AT-II-treated rats with L-NAME decreased DHE-derived fluorescence exclusively within the endothelium, thereby identifying eNOS as a significant source of superoxide. L-NAME increased the endothelial signal in vessel sections from AT-II/PETN-treated rats (like in the control group), indicating that PETN treatment was able to prevent eNOS uncoupling (Figure 3D and 3E). The AT-II/ISMN group displayed no difference compared with the AT-II group, indicating that eNOS uncoupling was not prevented by ISMN cotherapy. Similar beneficial effects of PETN but not ISMN were observed in SHR (Figure S3).



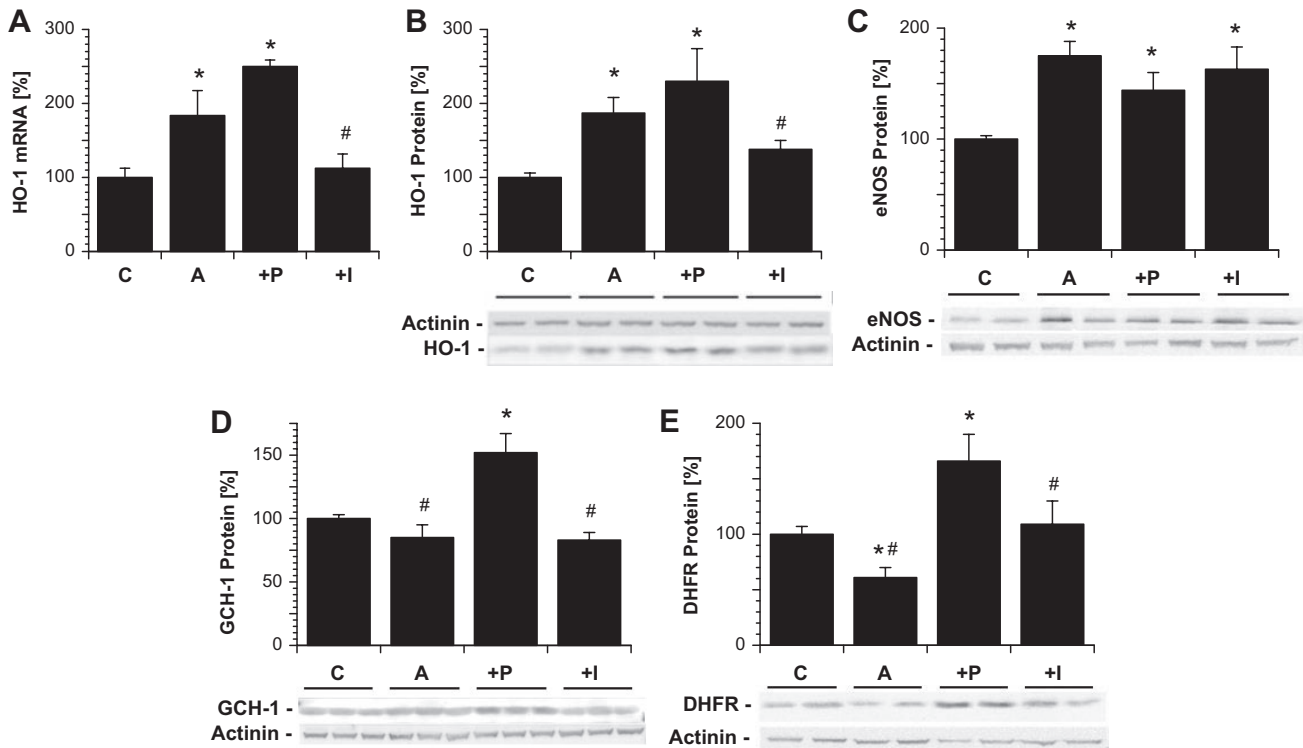
**Figure 3.** Effects of in vivo PETN and ISMN treatment on mitochondrial ROS formation (A and B), NADPH oxidase activity (A and C), and eNOS-dependent ROS formation (uncoupling; D and E) in AT-II rats. A, ROS formation in isolated cardiac mitochondria was measured by L-012 (100  $\mu\text{mol/L}$ ) ECL (left), and ROS production (NADPH oxidase activity) in membrane fractions from hearts was determined by lucigenin (5  $\mu\text{mol/L}$ )-ECL (right). B and C, ROS formation in isolated cardiac mitochondria and NADPH oxidase activity in membranous fractions were also quantified by 2-hydroxyethidium levels. The inserts show representative high-performance liquid chromatography chromatograms. E+ indicates ethidium; 2-HE, 2-hydroxyethidium. The data are mean  $\pm$  SEM of  $n=31$  to 43 (mitochondria and NADPH oxidase activity) experiments with tissue from  $\geq 10$  animals per group. The high-performance liquid chromatography data are mean  $\pm$  SEM of  $n=9$  (mitochondria) and  $n=15$  (NADPH oxidase activity) experiments with tissue from 3 to 5 animals per group. D and E, Fluorescence microscopy revealed ROS formation by red staining (top column). To determine eNOS-dependent ROS formation, vessels were preincubated with the NOS inhibitor L-NAME (bottom column). Pictures and data shown are representative for  $\geq 4$  animals per group. For methodological details see Figure S4 in the online Data Supplement.  $P < 0.05$ : vs \*control/DMSO; vs #AT-II+PETN. C indicates control; A, AT-II treated; P, AT-II and PETN treated; I, AT-II and ISMN treated.

### Effects of PETN and ISMN Cotreatment on Vascular Expression of eNOS, BH<sub>4</sub> Synthesizing Enzymes, and the Antioxidative Principle HO-1

AT-II treatment has been shown to increase aortic HO-1 gene expression at the mRNA and protein levels,<sup>22</sup> which was further increased by in vivo PETN treatment but decreased by ISMN cotherapy (Figure 4A and 4B). As before, we found a significant increase in the expression of eNOS in AT-II-treated rats, which was normalized by neither PETN nor ISMN (Figure 4C). It is important to note, however, that, in response to PETN treatment, eNOS was recoupled by PETN, whereas, in response to ISMN, the expression of an uncoupled eNOS was increased. AT-II infusion tended to decrease the expression of GCH-I and significantly decreased the levels of DHFR, another important enzyme for synthesis of BH<sub>4</sub> (the so-called rescue pathway for BH<sub>2</sub> (dihydrobiopterin) recycling; Figure 4D and 4E). PETN cotreatment significantly

increased expression of both BH<sub>4</sub> synthesizing enzymes even to higher levels as compared with the control identifying another important property of PETN, that is, how eNOS recoupling was achieved. The effects on BH<sub>4</sub> synthase and BH<sub>2</sub> (dihydrobiopterin) reductase were not shared by ISMN.

The role of HO-1 as the antioxidative principle of PETN was elucidated by 3 key experiments aiming to prove this hypothesis. The first experimental setup consisted of the treatment of control (HO-1<sup>+/+</sup>) and partially deficient (HO-1<sup>+/-</sup>) mice with PETN. In HO-1<sup>+/-</sup> but not HO-1<sup>+/+</sup> mice, PETN infusion induced tolerance against itself, envisaged by impaired vasodilator potency of the drug and increased mitochondrial ROS formation (Figure 5A and 5B). The second approach was on the basis of HO-1 induction by the known inducer of this enzyme, hemin, which improved AT-II-dependent endothelial dysfunction and prevented activation of NADPH oxidase (Figure 5C and 5D). The third



**Figure 4.** Effects of in vivo PETN and ISMN treatment on vascular HO-1 (mRNA and protein), eNOS, GCH-1, and DHFR expression in aortic tissue from hypertensive rats. Expressions of HO-1 mRNA (A) and HO-1 (B), eNOS (C), GCH-1 (D), and DHFR (E) protein were assessed by RT-PCR and Western blot analysis, respectively. Representative blots are shown at the bottom of the densitometric bar graphs. The data are mean  $\pm$  SEM of aortic rings from 6 to 8 animals per group.  $P < 0.05$ : vs \*control/DMSO; vs #AT-II+PETN. C indicates control; A, AT-II treated; P, AT-II and PETN treated; I, AT-II and ISMN treated.

experiment demonstrated that PETN did not improve endothelial dysfunction and cardiac oxidative stress in AT-II-treated HO-1<sup>+/-</sup> mice but further impaired vascular function and increased ROS formation in this setting (Figure 5E and 5F).

## Discussion

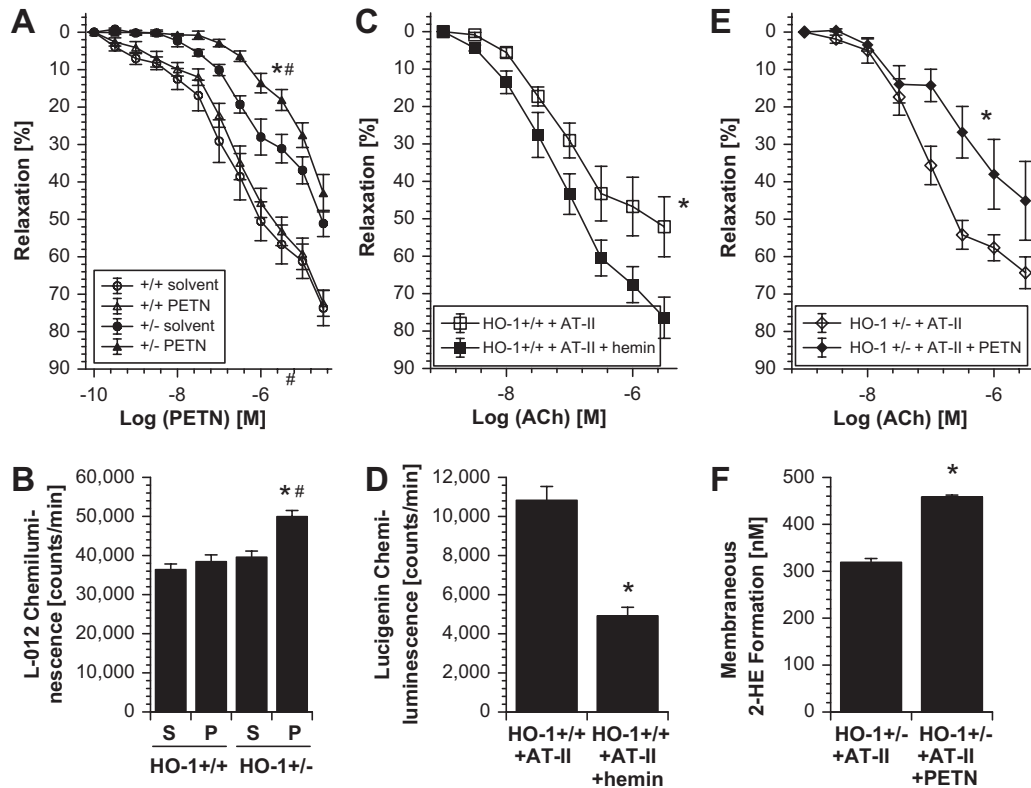
The present studies demonstrate that the organic nitrate PETN but not ISMN improves vascular function and reduces oxidative stress via inhibition of vascular superoxide production in mitochondria and by inhibition of the vascular NADPH oxidase in an experimental model of AT-II-induced hypertension. In almost all of the animal models where endothelial dysfunction is encountered, such as atherosclerosis,<sup>23</sup> chronic congestive heart failure,<sup>24</sup> AT-II-induced hypertension,<sup>4</sup> and diabetes mellitus,<sup>25</sup> we established that increased production of ROS via activation of the vascular NADPH oxidase and xanthine oxidase contributed considerably to this phenomenon. Interestingly, in all of the models, eNOS expression was upregulated rather than downregulated, suggesting that eNOS might be dysfunctional, as uncoupled under these circumstances.<sup>4,23–25</sup>

Previously, it was conceptualized that treatment with an exogenous source of NO (eg, GTN) could compensate for the diminished endothelial NO availability in atherosclerosis, thereby preventing the consequences of endothelial dysfunction, such as enhanced constriction and increased platelet aggregation. Theoretically, however, NO rapidly reacts with superoxide to produce the highly reactive intermediate, per-

oxynitrite, a potent oxidant that has been demonstrated to cause vascular (endothelial) dysfunction by inhibiting prostacyclin activity<sup>26</sup> and by causing eNOS uncoupling via oxidation of the important eNOS cofactor BH<sub>4</sub>.<sup>27</sup> Indeed, treatment of atherosclerotic animals with GTN worsened rather than improved endothelial function, caused consumption of plasma antioxidants such as  $\alpha$ - and  $\beta$ -carotene, decreased extracellular superoxide dismutase activity, and led to a dramatic increase in vascular protein tyrosine nitration as a footprint of peroxynitrite formation under GTN therapy.<sup>28</sup> In contrast to GTN, PETN is capable of upregulating the important antioxidant enzyme HO-1, which has been shown to play a key role for the prevention of nitrate tolerance and endothelial dysfunction under chronic GTN therapy.<sup>21</sup>

It remained to be established, however, whether PETN, a nitrate with antioxidant properties, is able to improve endothelial dysfunction in an animal model of endothelial dysfunction and oxidative stress. To address this issue, we used the model of AT-II infusion, in which the superoxide-producing enzymes are well characterized. For comparison, AT-II-treated animals were treated with the mononitrate ISMN. As before, infusion of AT-II led to a marked degree of endothelial dysfunction, as well as an attenuation of the endothelium-independent nitrovasodilator GTN associated with increased oxidative stress in vascular tissue. As superoxide sources, the NADPH oxidase, the mitochondria, and an uncoupled NOS were identified.

Experiments with isolated mitochondria and membrane fractions from the heart revealed that PETN and not ISMN



**Figure 5.** Effects of HO-1 deficiency vs HO-1 induction on vascular improvement by PETN. A and B, PETN treatment (75 mg/kg per day for 4 days) had no effect on PETN potency (PETN-induced relaxation) in aorta from control mice (HO-1<sup>+/+</sup>) but caused nitrate tolerance in aorta from mice with partial HO-1 deficiency (HO-1<sup>-/-</sup>). In accordance, cardiac mitochondrial ROS formation (L-012 ECL) was increased in PETN-treated HO-1<sup>-/-</sup> mice.  $P < 0.05$ ; vs \*HO-1<sup>+/+</sup>/DMSO; vs #HO-1<sup>-/-</sup>/DMSO. S indicates solvent; P, PETN treated. C and D, Hemin (25 mg/kg IP)-triggered HO-1 induction improved high-dose AT-II (1 mg/kg per day for 7 days)-induced endothelial dysfunction (ACh response) in aorta and NADPH oxidase activity in heart (lucigenin-ECL) from control mice (HO-1<sup>+/+</sup>).  $P < 0.05$ ; vs \*AT-II-treated HO-1<sup>+/+</sup>/DMSO. E and F, PETN (75 mg/kg per day for 7 days) failed to prevent endothelial dysfunction (ACh response) induced by low-dose AT-II (0.1 mg/kg per day for 7 days) in aorta from HO-1<sup>-/-</sup> mice. In accordance, PETN did not improve NADPH oxidase activity (2-HE formation by high-performance liquid chromatography analysis) in cardiac samples from AT-II-treated HO-1<sup>-/-</sup> mice.  $P < 0.05$ ; vs \*AT-II-treated HO-1<sup>-/-</sup>/DMSO. All of the data are mean  $\pm$  SEM of aortic rings and hearts from 4 to 5 animals per group.

treatment significantly reduced mitochondrial ROS production and to inhibit NADPH oxidase activity. Previously we have proposed that superoxide production by the vascular NADPH oxidase and mitochondria might represent so-called “kindling radicals,” which may react with NO to form peroxynitrite.<sup>25</sup> This intermediate in turn oxidizes BH<sub>4</sub> to the BH<sub>3</sub>· radical, thereby causing superoxide production by eNOS, the so called “bonfire” radical.<sup>27</sup> Thus, all of the measures that successfully reduce vascular superoxide production via inhibition of the NADPH oxidase should lead to a prevention of eNOS uncoupling. Indeed, as indicated by the DHE experiments with L-NAME, PETN but not ISMN prevented eNOS uncoupling in this model of oxidative stress. The observed reduction of mitochondrial ROS formation could be a direct consequence of decreased NADPH oxidase activity, because it was demonstrated recently that GTN-induced increases in NADPH oxidase activity can trigger mitochondrial ROS formation via K<sub>ATP</sub> (ATP-sensitive potassium) channels.<sup>11</sup>

Downregulation of the BH<sub>4</sub> synthesizing enzyme DHFR has been proposed to contribute substantially to endothelial dysfunction and eNOS uncoupling in the AT-II infusion model.<sup>29</sup> In 2 recent studies with diabetic rats, we were able

to demonstrate that the prevention of eNOS uncoupling in response to atorvastatin or the Ang II type 1 receptor blocker telmisartan was at least in part secondary to an upregulation of BH<sub>4</sub> synthesizing enzyme, such as the GCH-I or the DHFR.<sup>9,10</sup> With the present studies, we established that PETN and not ISMN was able to substantially upregulate not only DHFR but also GCH-I, which may also contribute considerably to the recoupling of the enzyme.

As mentioned before, eNOS protein was upregulated rather than downregulated in this animal model of hypertension. It is important to note that this is likely attributed to increased H<sub>2</sub>O<sub>2</sub> production, which has been shown previously to increase eNOS expression at the transcriptional and translational levels.<sup>30</sup> Thus, a reduction in vascular oxidative stress should always result in a normalization of eNOS under these circumstances. With the present studies we were able to show that AT-II-upregulated eNOS was not modified by PETN or ISMN treatment. The fact, however, that, in response to PETN but not ISMN treatment, eNOS was recoupled explains why PETN treatment and not ISMN treatment improved endothelial dysfunction.

In one of our recent articles we identified the antioxidant enzyme HO-1 as the key player determining whether an

organic nitrate causes endothelial dysfunction and nitrate tolerance.<sup>21</sup> HO-1 exerts its beneficial effects on vascular function via formation of the sGC stimulator carbon monoxide, the antioxidant bilirubin, and the chelator protein of free iron, ferritin.<sup>31</sup> Likewise, with the present studies we can show that AT-II upregulated HO-1 expression at the mRNA and protein levels, which was further stimulated by PETN but not by ISMN treatment. The results by using HO-1-deficient mice (HO-1<sup>+/-</sup>) clearly demonstrate that HO-1 largely contributes to the pleiotropic protective properties of PETN, because the beneficial effects of PETN on endothelial dysfunction in the AT-II hypertension model were almost completely abolished by heterozygous HO-1 deficiency. Otherwise, induction of HO-1 by hemin was able to prevent vascular dysfunction by high-dose AT-II treatment of control mice (HO-1<sup>+/+</sup>).

To address whether similar effects can be observed in a genetically determined model of hypertension, SHR were studied. The results confirm those obtained from the AT-II infusion model. PETN but not ISMN treatment markedly reduced vascular superoxide production, as quantified by DHE staining and by lucigenin-ECL. The effects of PETN on vascular function in SHR were significantly different from those of ISMN although less pronounced as compared with the effects in AT-II-triggered hypertension. For detailed consideration of these differences see the Extended Results section in the online Data Supplement. Therefore, the observations on the beneficial effects of PETN in both animal models of arterial hypertension are in accordance with previous studies, indicating that PETN improves experimental atherosclerosis in rabbits.<sup>32,33</sup> It should be noted that these authors have also reported on antiatherosclerotic effects of ISMN and improvement of endothelial dysfunction by this drug,<sup>34,35</sup> which, however, is at variance with our present observations and a human study indicating detrimental effects of ISMN on endothelial function in healthy volunteers.<sup>13</sup>

## Perspectives

The results of the present studies show for the first time that, in an animal model of oxidative stress, the organic nitrate PETN but not the mononitrate ISMN is able to improve endothelial dysfunction. PETN substantially inhibited NADPH oxidase activity, inhibited mitochondrial superoxide production, and prevented eNOS uncoupling. The recoupling of eNOS may be a consequence of a reduction of oxidative stress secondary to upregulated HO-1 protein but may also be attributed to PETN-mediated upregulation of the key enzymes for BH<sub>4</sub> synthesis, such as the GCH-I and the DHFR. These preclinical studies contribute to the understanding of why PETN treatment does not cause tolerance or endothelial dysfunction. The spectrum of antioxidant features of this compound indicates that PETN may not be used only for the treatment of symptomatic coronary artery disease but also to beneficially influence the progression of the atherosclerotic process.

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