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

Swenja Schuhmacher, Philip Wenzel, Eberhard Schulz, Matthias Oelze, Christian Mang, Jens Kamuf, Tommaso Gori, Thomas Jansen, Maike Knorr, Susanne Karbach, Marcus Hortmann, Falk Mäthner, Aruni Bhatnagar, Ulrich Förstermann, Huige Li, Thomas Münzel, Andreas Daiber

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. 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. ROS sources involved may include the NADPH oxidases, an uncoupled endothelial NO synthase (NOS; eNOS), and mitochondrial superoxide sources. 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, whereas blood pressure responses to AT-II were reduced in NADPH oxidase 1–deficient mice. Increased vascular ROS production and endothelial dysfunction are also accompanied by increased eNOS expression but decreased vascular NO production. 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 and identified the downregulation of tetrahydrobiopterin (BH4) synthesizing enzymes GTP-cyclohydrolase-I (GCH-I) and dihydrofolate reductase (DHFR) as key events for the development of endothelial dysfunction.

Organic nitrates act as endothelium-independent vasodilators of coronary arteries, venous capacity vessels, and collaterals. Nitroglycerin (GTN) is one of the most widely used agents of coronary vasodilation. In contrast to isosorbide-5-mononitrate, as an organic nitrate able to improve rather than to worsen endothelial function, 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 (GCH-I) and dihydrofolate reductase (DHFR) as key events for the development of endothelial dysfunction. NADPH oxidase 1–deficient mice.

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From the Johannes Gutenberg University Hospital (S.S., P.W., E.S., M.O., J.K., T.G., T.J., M.K., S.K., F.M., T.M., A.D.), 2nd Medical Clinic, Molecular Cardiology, Mainz, Germany; Institut für Pharmakologie (C.M., M.H., U.F., H.L.), Johannes Gutenberg-Universität Mainz, Mainz, Germany; Experimental Research Laboratory (A.B.), Division of Cardiology, University of Louisville and Jewish Hospital Heart and Lung Institute, Louisville, Ky. S.S. and P.W. and T.M. and A.D. contributed equally to this study and should therefore be considered as joint first and senior authors.

Correspondence to Andreas Daiber, Universitätsmedizin der Johannes Gutenberg Universität, II. Med Klinik u Poliklinik-Labor für Molekulare Kardiologie, Obere Zahlbacher Str. 63, 55101 Mainz, Germany. E-mail daiber@uni-mainz.de

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mitochondrial aldehyde dehydrogenase 2. These findings though both compounds are clearly not bioactivated by the ALDH2, do not induce tolerance or endothelial dysfunction,12,17 but do cause nitrate tolerance and endothelial dysfunction,13 aldehyde dehydrogenase 2,12 does not induce tolerance or stimulate vascular superoxide production. For instance, treatment with the nitrate PETN, while still undergoing mitochondrial activation by the aldehyde dehydrogenase 2,12 does not induce tolerance or stimulate vascular superoxide production. However, GTN caused a strong oxidant enzyme heme oxygenase (HO)-1, which preserved endothelial dysfunction. Recent data indicate that GTN-induced ROS formation accounts for both phenomena, as well as the development of nitrate tolerance and endothelial dysfunction.6

Methods and Materials

Materials

PETN was obtained from Actavis. GTN was used from a Nitrolingual 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. 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−/− or HO-1+/+ mice on a 129sv×BALB/c background were treated with PETN (75 mg/kg per day; DMSO, 0.5 μL/h) for 7 days, 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-preconstricted aortic ring segments, as described previously. Murine aorta were preconstricted by phenylephrine (10 μmol/L). Tissues were divided into two experimental groups. A group of mice was treated with PETN (15 mg/kg per day) or ISMN (75 mg/kg per day) versus vehicle (dimethyl sulfoxide [DMSO]). Male HO-1−/− or HO-1+/+ mice were treated with PETN (75 mg/kg per day; DMSO, 0.5 μL/h) for 7 days, 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.

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. 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.
Western Blot Analysis and RT-PCR

Western blotting against eNOS, GCH-1, DHFR, and HO-1 was performed as described previously. 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. For details see the Extended Methods section in the online Data Supplement.

Statistical Analysis

Results are expressed as mean±SEM. pD2 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 Cotreatment on Vascular and Cardiac ROS Production, as Well as eNOS Uncoupling in AT-II–Induced Hypertension

Weight gain in solvent-treated control animals was 50±6 g in 1 week, whereas AT-II treatment caused weight loss of 61±16 g. Weight loss in AT-II–treated animals was significantly improved in hypertensive rats by PETN cotherapy (3±17 g), whereas ISMN treatment had no significant effect (~13±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 BH4 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 SHRs are valid models of experimental hypertension. The beneficial effects of PETN and sepiapterin on vascular function were almost absent in SHRs, 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, Nω-nitro-L-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 SHRs (Figure S3).
Effects of PETN and ISMN Cotreatment on Vascular Expression of eNOS, BH₄ 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, 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 BH₄ (the so-called rescue pathway for BH₂ (dihydrobiopterin) recycling; Figure 4D and 4E). PETN cotreatment significantly increased expression of both BH₄ 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 BH₄ synthase and BH₂ (dihydrobiop- terin) 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⁻/⁻) and partially deficient (HO-1⁺/⁻) mice with PETN. In HO-1⁻/⁻ but not HO-1⁺/⁻ 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
experiment demonstrated that PETN did not improve endothelial dysfunction and cardiac oxidative stress in AT-II–treated HO-1/H11001/H11002 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,23 chronic congestive heart failure,24 AT-II–induced hypertension,4 and diabetes mellitus,25 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.4,23–25

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, peroxynitrite, a potent oxidant that has been demonstrated to cause vascular (endothelial) dysfunction by inhibiting prostacyclin activity26 and by causing eNOS uncoupling via oxidation of the important eNOS cofactor BH4.27 Indeed, treatment of atherosclerotic animals with GTN worsened rather than improved endothelial function, caused consumption of plasma antioxidants such as α- and β-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.28 In contrast to GTN, PETN is capable of upregulating the important antioxidant enzyme HO-I, which has been shown to play a key role for the prevention of nitrate tolerance and endothelial dysfunction under chronic GTN therapy.21

It remained to be established, however, whether PETN, a nitrate with antioxidant properties, is able to improve endothelial function 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
mitochondrial ROS formation via KATP. (ATP-sensitive potassium) channels. 

This intermediate in turn oxidizes BH4 to the "kindling radicals," which may react with NO to form peroxynitrite. This intermediate is likely attributed to increased H2O2 production, which has been shown previously to increase eNOS expression at the transcriptional and translational levels. 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.

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 H2O2 production, which has been shown previously to increase eNOS expression at the transcriptional and translational levels. 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.\textsuperscript{21} 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.\textsuperscript{31} 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\textsuperscript{−/−}) 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\textsuperscript{+/−}).

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 SHRs 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.\textsuperscript{32,33} It should be noted that these authors have also reported on antiatherosclerotic effects of ISMN and improvement of endothelial dysfunction by this drug.\textsuperscript{34,35} which, however, is at variance with our present observations and a human study indicating detrimental effects of ISMN on endothelial function in healthy volunteers.\textsuperscript{13}

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\textsubscript{4} synthesis, such as the GCH-1 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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Disclosures

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Pentaerithritol tetranitrate improves angiotensin II induced vascular dysfunction via induction of heme oxygenase-1

Swenja Schuhmacher*1, Philip Wenzel*1, Eberhard Schulz1, Matthias Oelze1, Christian Mang2, Jens Kamuf1, Tommaso Gori1, Thomas Jansen1, Maike Knorr1, Susanne Karbach1, Marcus Hortmann2, Falk Mäthner1, Aruni Bhatnagar3, Ulrich Förstermann2, Huige Li2, Thomas Münzel1, and Andreas Daiber†1
Extended Methods

Animal models, in vivo infusion of angiotensin-II and SHR
All animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the U.S. National Institutes of Health and was granted by the Ethics Committee of the University Hospital Mainz. Male Wistar rats (60 in total; weight 250g; Charles River, Sulzfeld, Germany) were anesthetized by isoflurane inhalation and treated with a subcutaneous osmotic minipump (Durect Corp., Cupertino, CA 95014) containing either AT-II (1.0mg/kg/d) or solvent (0.9 % NaCl) for 7d, as described previously 1. Male SHR (20 in total; 6 months of age) and Wistar-Kyoto control rats were obtained from Charles River Laboratories (Sulzfeld, Germany). Animals from both groups were randomized to receive either PETN (15mg/kg/d), ISMN (75mg/kg/d) or vehicle (DMSO) via an additional subcutaneous osmotic minipump. After 7d the rats were sacrificed under isoflurane anesthesia. Male HO-1+/+ or HO-1-/- mice (3-4 months old) on a 129sv x BALB/c mixed genetic background as described 2, were treated with a subcutaneous osmotic minipump containing either PETN (75mg/kg/d) or solvent (DMSO, 0.5µl/h) for 7d 3. The mice were generated as previously reported. Male HO-1+/+ were also treated with high dose AT-II (1.0mg/kg/d) or solvent (0.9 % NaCl) for 7d and the HO-1 inducer hemin (25mg/kg single i.p. injection, 12h prior to sacrifice). Male HO-1-/- were also treated with low dose AT-II (0.1mg/kg/d) or solvent (0.9 % NaCl) and co-treated with either PETN (75mg/kg/d) or solvent (DMSO, 0.5µl/h) for 7d.

Determination of blood pressure
Systolic blood pressure was obtained on a weekly basis in isoflurane anesthetized rats using a tail cuff non-invasive blood pressure system coupled to a PowerLab system (ML125 NIBP, ADInstruments, Colorado Springs, CO). A minimum of three measurements were obtained from each rat. We used a protocol that was previously published 4.

Western Blot analysis and RT-PCR
Isolated aortic tissue was frozen and homogenized in liquid nitrogen. Proteins were separated by SDS-PAGE and blotted onto nitrocellulose membranes. After blocking, immunoblotting was performed with antibodies against α-actinin (100kDa) or actin (42kDa) (1:2500, Sigma-Aldrich) as controls for loading and transfer, eNOS (1:1000, BD Biosciences, USA), GTP-cyclohydrolase-1 (GCH-1: 1µg/ml, Abnova Corp., Germany), dihydrofolate reductase (DHFR: 1µg/ml, RDI Div. of Fitzgerald Ind., USA) 5 and HO-1 (1:5000, monoclonal, Stressgen, San Diego, CA) 6. Detection was performed by ECL with peroxidase conjugated anti–rabbit/mouse (1:10000, Vector Lab., Burlingame, CA) and anti-goat (1:5000, Santa Cruz Biotechnologies, USA) secondary antibodies. The antibody-specific bands were quantified by densitometry as described. 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). TaqMan® Gene Expression assays (Applied Biosystems, Foster City, CA) for HO-1 and GAPDH were purchased as probe and primer sets and gene expression was normalized to the endogenous control, GAPDH mRNA as described6.

Assessment of vascular and cardiac oxidative stress
Mitochondria were isolated from heart and mitochondrial ROS formation was detected by L-012 (100µM)-enhanced chemiluminescence (ECL) in the presence of succinate (5mM) as previously described 7. Membrane fractions were isolated from heart and NADPH oxidase activity was determined by lucigenin (5µM) ECL in the presence of NADPH (200µM) according to a previous protocol. Vascular ROS formation was detected in intact aortic ring
Segments (0.5 cm length) by lucigenin (5µM) ECL and dihydroethidine (1µM)-dependent fluorescence in aortic cryo-sections (fluorescent microtopography) as reported elsewhere. The functional state of eNOS (coupled or uncoupled) was estimated from dihydroethidine-treated aortic cryo-sections in the presence and absence of the NOS inhibitor L-NAME (0.5mM) by fluorescence microscopy as described. Mitochondrial ROS production and NADPH oxidase activity were determined by HPLC-based quantification of the conversion of dihydroethidine to 2-hydroxyethidium as described.

**Extended Results**

**Effects of PETN and ISMN co-treatment on vascular function and ROS production in SHR rats**

Spontaneously hypertensive rats (SHR) had significantly higher levels of aortic ROS formation as compared to corresponding controls (Wistar-Kyoto rats), which was demonstrated by dihydroethidine-dependent fluorescent microtopography in aortic cryo-sections as well as lucigenin ECL in intact aortic ring segments (Figure S5 A-C). Both methods revealed a significant improvement of vascular ROS formation in SHR by PETN but not ISMN therapy.

SHR rats had significantly impaired endothelial and smooth muscle function as compared to WKY controls (Figure S2). The effects of PETN on endothelium-dependent (ACh) and –independent (GTN) relaxation in SHR were not as pronounced as those on vascular oxidative stress but still reflected the tendency observed in the ROS determination assays (compare Figure S2 versus S3). Although the improvement of endothelial and smooth muscle function was not significant under PETN therapy, the impairment under ISMN was significant as compared to the SHR+PETN group (Figure S2). These rather small effects of PETN therapy on vascular function may be attributed to the experimental setup. We started the organic nitrate therapy in 6 months old SHR rats. These animals already developed severe hypertension (Figure S1) and vascular remodeling may interfere with pronounced effects of cardiovascular therapeutics as previously observed for statin treatment of SHR. Most studies on antihypertensive therapy in SHR rats start in young animals, in the prehypertensive state to prevent vascular remodeling and development of hypertension and its adverse effects. Therefore, SHR may not represent the best experimental model of hypertension to study improvement of vascular dysfunction by cardiovascular therapeutics. This consideration is further supported by the weak effect of BH4/SOD pretreatment on endothelial dysfunction in aorta from SHR as compared to the significant beneficial effect of the eNOS cofactor on endothelial dysfunction in aorta from AT-II-infused rats (compare Figure S2 versus Figure 1 in the main manuscript). Obviously, endothelial dysfunction in SHR is rather not dependent on eNOS dysfunction but on structural changes in the vascular wall. However, in a recent study Dovinova et al. have demonstrated that endothelial function of aorta from SHR was improved by treatment with PETN for 6 weeks. It should be noted that these authors used a 6.5-fold higher dose of PETN (100 mg/kg/d) than used in the present study and treatment was maintained for 6 weeks instead of 1 week.

**Effects of PETN and ISMN co-treatment on renal salt handling in SHR and AT-II infused rats**

As known from Dahl salt-sensitive rats, renal salt handling may largely affect blood pressure. Also in SHR, effects of high salt diet on blood pressure have been observed. Since there is...
no literature available on the effects of organic nitrates on renal salt handling, we cannot exclude that changes in renal salt handling may contribute to the beneficial effects observed for PETN and likely to the adverse effects of ISMN. This assumption is supported by modulation of renal salt handling by nitric oxide.

Extended References


Table S1. Effects of treatment with PETN or ISMN on the potency and efficacy of endothelium-dependent (ACh) and -independent (GTN) vasodilators in isolated aortic segments from hypertensive rats.

| In vivo Treatment     | Potency (pD$_2$)$^|$ | Efficacy (%) |
|----------------------|----------------------|--------------|
|                      | ACh                  | GTN          | ACh | GTN |
| Control + DMSO       | 7.30±0.05 (n=41)     | 7.66±0.04 (n=43) | 90±1 | 99±0 |
| AT-II + DMSO         | 6.79±0.07* (n=57)    | 7.24±0.06* (n=53) | 58±3* | 98±1 |
| AT-II + PETN         | 6.93±0.06* (n=46)    | 7.51±0.06*† (n=50) | 70±2†  | 99±0 |
| AT-II + ISMN         | 6.54±0.07*‡ (n=36)   | 7.16±0.06*‡ (n=34) | 61±3*‡  | 97±1*‡ |

* P<0.05 vs. control; † P<0.05 vs. AT-II; ‡ P<0.05 vs. AT-II + PETN. 
$^|$ Potency is –log EC$_{50}$ and efficacy is defined as maximal relaxation obtained with the highest employed concentration of the vasodilator. n indicates the number of aortic rings per group.
Table S2. Effects of treatment with PETN or ISMN on sensitivity to vasoconstrictors in isolated aortic segments from hypertensive rats.

<table>
<thead>
<tr>
<th>In vivo Treatment</th>
<th>Induced Vasoconstriction [g]</th>
<th>KCl</th>
<th>Phenylephrine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control + DMSO</td>
<td></td>
<td>3.54±0.16 (n=30)</td>
<td>2.48±0.26 (n=29)</td>
</tr>
<tr>
<td>AT-II + DMSO</td>
<td></td>
<td>3.60±0.24 (n=28)</td>
<td>2.91±0.35 (n=30)</td>
</tr>
<tr>
<td>AT-II + PETN</td>
<td></td>
<td>3.41±0.24 (n=30)</td>
<td>2.78±0.27 (n=28)</td>
</tr>
<tr>
<td>AT-II + ISMN</td>
<td></td>
<td>3.25±0.24 (n=28)</td>
<td>2.77±0.23 (n=30)</td>
</tr>
</tbody>
</table>

* 80 mM KCl and 300 nM phenylephrine. n indicates the number of aortic rings per group.
Figure S1. Left panel: Systolic blood pressure in control, AT-II-treated rats or SHR was measured by the tail cuff method. Data shown are the mean±SEM of at least 4 measurements. P < 0.05: * vs. Ctr. Right panel: Weight gain of rats in the different treatment groups (initial weight was 250g). Data shown are the mean±SEM of at least 8 measurements. P < 0.05: * vs. Ctr; + vs. AT-II.

Figure S2. Effects of in vivo pentaerythrityl tetranitrate (PETN) and isosorbide-5-mononitrate (ISMN) treatment on the vasoreactivity of aorta from spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) controls. Endothelium-dependent (ACh, A) or –independent (GTN, B) vasodilation was assessed by isometric tension recording. (C) The effect of sepiapterin (100 µM), a BH4 precursor, and PEG-SOD (100 U/ml) preincubation of aortic rings from SHR for 1 h was determined in separate experiments. Data shown are representative for at least 4 animals/group. P < 0.05: * vs. WKY+DMSO; # vs. SHR+PETN. The statistics were based on 1-way-ANOVA comparison of pD2-values and efficacies but also on comparisons of all concentrations in all groups by 2-way-ANOVA analysis (for sake of clarity significance is not shown for all data points).
Figure S3. Effects of in vivo pentaerithrityl tetranitrate (PETN) and isosorbide-5-mononitrate (ISMN) treatment on the reactive oxygen species formation in aorta from spontaneously hypertensive rats (SHRs) and Wistar-Kyoto (WKY) controls. Dihydroethidine (DHE, 1µM) staining of aortic cryo-sections (A), densitometric quantification of the DHE-derived reactive oxygen species signal throughout the vessel wall (B) and lucigenin (5µM) enhanced chemiluminescence (ECL) in intact aortic ring segments (C). Pictures and data shown are representative for 3-4 animals/group. P < 0.05: * vs. Ctr/DMSO; # vs. AT-II+PETN. W, Wistar-Kyoto controls; S, spontaneously hypertensive rats; P, PETN-treated SHRs; I, ISMN-treated SHRs.
Figure S4. Effects of PETN treatment on eNOS uncoupling. Effects of in vivo pentaerithrityl tetranitrate (PETN) and isosorbide-5-mononitrate (ISMN) treatment on eNOS-dependent reactive oxygen species formation (uncoupling) in aorta from hypertensive rats. Dihydroethidine (DHE, 1µM)-fluorescent microtopography was used to assess vascular reactive oxygen species formation in aortic cryo-sections which were incubated with dihydroethidine. (A) eNOS uncoupling was assessed by densitometric quantification of DHE staining in the endothelial cell layer which was extracted from the whole microscope image. (B) A fixed area was used for densitometric quantification and the procedure is shown for endothelial cell layer of AT-II (#2) image. eNOS uncoupling was previously assessed by the effects of L-NAME on DHE staining $^{1,5}$. The method of densitometric quantification of endothelial DHE staining was adopted from the protocol of Alp et al. $^{16}$.