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

Swenja Schuhmacher<sup>\*,1</sup>, Philip Wenzel<sup>\*,1</sup>, Eberhard Schulz<sup>1</sup>, Matthias Oelze<sup>1</sup>, Christian Mang<sup>2</sup>, Jens Kamuf<sup>1</sup>, Tommaso Gori<sup>1</sup>, Thomas Jansen<sup>1</sup>, Maïke Knorr<sup>1</sup>, Susanne Karbach<sup>1</sup>, Marcus Hortmann<sup>2</sup>, Falk Mäthner<sup>1</sup>, Aruni Bhatnagar<sup>3</sup>, Ulrich Förstermann<sup>2</sup>, Huige Li<sup>2</sup>, Thomas Münzel<sup>1</sup>, and Andreas Daiber<sup>†,1</sup>

<sup>1</sup>Johannes Gutenberg University Hospital, <sup>2</sup>nd Medical Clinics, Molecular Cardiology, Mainz, Germany

<sup>2</sup>Institut für Pharmakologie, Johannes Gutenberg-Universität Mainz, 55131 Mainz

<sup>3</sup>Experimental Research Laboratory, Division of Cardiology, University of Louisville and Jewish Hospital Heart and Lung Institute, Louisville, Ky, USA

### Abstract

The organic nitrate pentaerithryl tetranitrate treatment is devoid of nitrate tolerance, which has been attributed to the induction of the antioxidant enzyme heme oxygenase-1. With the present study we tested, whether chronic treatment with pentaerithryl tetranitrate can improve angiotensin-II induced vascular oxidative stress and dysfunction. In contrast to isosorbide-5-mononitrate (75mg/kg/d/7d), treatment with pentaerithryl tetranitrate (15mg/kg/d/7d) improved the impaired endothelial and smooth muscle function and normalized vascular and cardiac reactive oxygen species production (mitochondria, NADPH oxidase activity and uncoupled endothelial nitric oxide synthase) as assessed by dihydroethidine staining, lucigenin-enhanced chemiluminescence and quantification of dihydroethidine oxidation products in angiotensin-II (1mg/kg/d/7d) treated rats. The antioxidant features of pentaerithryl tetranitrate were recapitulated in spontaneously hypertensive rats. In addition to increase in heme oxygenase-1 protein expression, pentaerithryl tetranitrate but not isosorbide-5-mononitrate normalized vascular reactive oxygen species formation, augmented aortic protein levels of the tetrahydrobiopterin-synthesizing enzymes GTP-cyclohydrolase-I and dihydrofolate reductase in angiotensin-II treated rats, thereby preventing endothelial nitric oxide synthase uncoupling. Knockout of heme oxygenase-1 completely abolished the beneficial effects of pentaerithryl tetranitrate in angiotensin-II treated mice, whereas heme oxygenase-1 induction by hemin (25mg/kg) mimicked the effect of pentaerithryl tetranitrate. Improvement of vascular function in this particular model of arterial hypertension by pentaerithryl tetranitrate largely depends on the induction of the antioxidant enzyme heme oxygenase-1 and identifies pentaerithryl tetranitrate, in contrast to isosorbide-5-mononitrate, as an organic nitrate being able to improve rather than to worsen endothelial function.

<sup>†</sup>Address for correspondence: Prof. Dr. Andreas Daiber Universitätsmedizin der Johannes Gutenberg Universität, II. Med. Klinik u. Poliklinik – Labor für Molekulare Kardiologie, Obere Zahlbacher Str. 63, 55101 Mainz, Germany Tel: +49 (0)6131 3933301; Fax: +49 (0)6131 3933304; daiber@uni-mainz.de.

\*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 author

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## Keywords

pentaerythrityl tetranitrate; isosorbide-5-mononitrate; angiotensin-II; SHR; endothelial dysfunction; vascular oxidative stress

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## Introduction

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 nitric oxide synthase (eNOS)<sup>4</sup> as well as 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 Nox1 overexpression in transgenic mice potentiates AT-II-induced hypertension<sup>6</sup> while blood pressure responses to AT-II were reduced in Nox1-deficient mice<sup>7</sup>. Increased vascular ROS production and endothelial dysfunction are also accompanied by increased eNOS expression but decreased vascular nitric oxide (NO) production<sup>8</sup>. Recently, we were able to demonstrate that pharmacological intervention with a statin or an AT<sub>1</sub>-receptor-blocker improved vascular dysfunction and reduced oxidative stress in an experimental model of diabetes mellitus<sup>9, 10</sup> and identified the down-regulation 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 anti-ischemic drugs for more than a century. The chronic efficacy of nitrates, however, is blunted due to side effects such as the development nitrate tolerance and endothelial dysfunction. Recent data indicate that GTN-induced ROS formation accounts for both phenomena since ROS formed in response to GTN therapy since both phenomena can be corrected by treatment with antioxidants<sup>11, 12</sup>. Treatment with mono- and dinitrates also causes tolerance and endothelial dysfunction<sup>13</sup>, although both compounds are clearly not bioactivated by the mitochondrial aldehyde dehydrogenase (ALDH-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 mono- and dinitrates<sup>15</sup>. Among all organic nitrates, the most frequently used compounds in the treatment of coronary artery disease comprise GTN, pentaerythrityl tetranitrate (PETN; in Germany), isosorbide dinitrate (ISDN) and isosorbide-5-mononitrate (ISMN; USA).

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 pentaerythrityl tetranitrate (PETN), while still undergoing mitochondrial activation by the ALDH-2, does not induce tolerance or stimulate vascular superoxide production<sup>12</sup>. While GTN caused a strong oxidative stress mediated inhibition of the mitochondrial ALDH-2, PETN induced an upregulation of the antioxidant enzyme HO-1, which preserved ALDH-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> and endothelial dysfunction<sup>17</sup>.

Based on 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 also tolerance development.

## Methods and Materials

### Materials

PETN was obtained from Actavis (Langenfeld, Germany). GTN was used from a Nitrolingual infusion solution (Pohl-Boskamp, Hohenlockstedt, Germany). All other chemicals were purchased from Sigma-Aldrich, Merck or Fluka.

### 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 (250g) were treated with either AT-II (1.0mg/kg/d) or solvent (0.9% NaCl) for 7d, as described previously<sup>18</sup>. Male SHR and Wistar-Kyoto control rats were obtained from Charles River Laboratories (Sulzfeld, Germany). Animals from both groups were treated with either PETN (15mg/kg/d), ISMN (75mg/kg/d) versus vehicle (DMSO). Male HO-1<sup>+/+</sup> or HO-1<sup>+/-</sup> mice on a 129sv × BALB/c background were treated with either PETN (75mg/kg/d), (DMSO, 0.5μl/h) for 7d<sup>19</sup>, high dose AT-II (1.0mg/kg/d) versus solvent (0.9 % NaCl) for 7d with and without the HO-1 inducer hemin (25mg/kg single i.p. injection, 12h prior to sacrifice) or low dose AT-II (0.1mg/kg/d) versus solvent (0.9 % NaCl) with or without PETN (75mg/kg/d) for 7d. For details see “Extended Methods” 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 nitroglycerin (GTN) were determined in organ chambers by isometric tension studies using phenylephrine-precontracted aortic ring segments, as previously described<sup>18</sup>. Murine aorta were precontracted by prostaglandin F<sub>2α</sub>, as previously published<sup>19</sup>–<sup>20</sup>.

### Assessment of vascular and cardiac oxidative stress

Vascular and cardiac oxidative stress was assessed by L-012 (a luminol derivative)- or lucigenin-enhanced chemiluminescence (ECL) and dihydroethidine-dependent fluorescence as described elsewhere<sup>10</sup>–<sup>14</sup>–<sup>18</sup>–<sup>21</sup>. For details see “Extended Methods” in the online data supplement available at <http://hyper.ahajournals.org>. For determination of cardiac ROS in mice, 2–3 hearts were pooled, mitochondria and membrane fractions were isolated as recently published<sup>11</sup>–<sup>19</sup>.

### Western Blot analysis and RT-PCR

Western blotting against eNOS, GCH-1, DHFR and HO-1 was performed as previously described<sup>10</sup>–<sup>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, 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 described<sup>21</sup>. For details see “Extended Methods” in the online data supplement available at <http://hyper.ahajournals.org>.

## Statistical Analysis

Results are expressed as means $\pm$ SEM. pD<sub>2</sub>-values (potencies) were obtained by logit-transformation. One way ANOVA (with Bonferroni's or Dunn's correction for comparison of multiple means) was used for comparisons of vasodilator potency and efficacy; vascular and cardiac ROS formation as well as aortic protein and mRNA expression. P values <0.05 were considered significant.

## Results

### Effects of PETN and ISMN co-therapy on AT-II-induced vascular dysfunction

Weight gain in solvent-treated control animals was 50 $\pm$ 6g in one week whereas AT-II treatment caused weight loss of 61 $\pm$ 16g. Weight loss in AT-II treated animals was significantly improved in hypertensive rats by PETN co-therapy (3 $\pm$ 17g), while ISMN treatment had no significant effect (-13 $\pm$ 18g). In vivo treatment with PETN rather improved endothelial dysfunction in AT-II-treated animals (Figure 1A and Table S1 in the online data supplement available at <http://hyper.ahajournals.org>), while ISMN further impaired AT-II-induced endothelial dysfunction AT-II infusion-induced cross tolerance to GTN was corrected by PETN co-treatment, but not by ISMN (Figure 1B and Table S1). Treatment of vessels from AT-II-infused rats with the BH<sub>4</sub> precursor sepiapterin normalized endothelial function (Figure 1C). Sensitivity of aorta to vasoconstriction by KCl and phenylephrine was not changed (Table S2 in the online data supplement available at <http://hyper.ahajournals.org>). Blood pressure data are presented in Figure S1 in the online data supplement available at <http://hyper.ahajournals.org> 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, in the online data supplement available at <http://hyper.ahajournals.org>).

### Effects of PETN and ISMN co-treatment on vascular and cardiac ROS production as well as eNOS uncoupling in AT-II-induced hypertension

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

To assess the contribution of an uncoupled eNOS to superoxide formation due to eNOS uncoupling, rat aortic tissue was incubated with a NOS inhibitor, N<sup>G</sup>-nitro-L-arginine methylester (L-NAME). L-NAME increased dihydroethidine (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 E). The AT-II/

ISMN group displayed no difference compared to the AT-II group, indicating that eNOS uncoupling was not prevented by ISMN co-therapy.

Similar beneficial effects of PETN but not ISMN were observed in SHR (Figure S3, in the online data supplement available at <http://hyper.ahajournals.org>).

### **Effects of PETN and ISMN co-treatment on vascular expression of eNOS, tetrahydrobiopterin synthesizing enzymes and the antioxidative principle HO-1**

Angiotensin-II treatment has been shown to increase aortic HO-1 gene expression at the mRNA and protein level<sup>22</sup>, which was further increased by in vivo PETN treatment, but decreased by ISMN co-therapy (Figure 4A and B). As before, we found a significant increases in the expression of eNOS in AT-II-treated rats, which was neither normalized by PETN nor ISMN (Figure 4C). It is important to note, however, that in response to PETN treatment, eNOS was recoupled by PETN while in response to ISMN, the expression of an uncoupled eNOS was increased. AT-II infusion tended to decrease the expression of GTP-cyclohydrolase-I (GCH-I) and significantly decreased the levels of dihydrofolate reductase (DHFR) another important enzyme for synthesis of BH<sub>4</sub> (the so-called rescue pathway for BH<sub>2</sub> recycling, Figure 4D and E). PETN co-treatment significantly increased expression of both BH<sub>4</sub> synthesizing enzymes even to higher levels as compared to the control identifying another important property of PETN, how eNOS recoupling was achieved. The effects on BH<sub>4</sub> synthase and BH<sub>2</sub> reductase were not shared by ISMN.

The role of HO-1 as the antioxidative principle of PETN was elucidated by three 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 B). The second approach was based on 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 D). The third experiment demonstrated that PETN did not improve endothelial dysfunction and cardiac oxidative stress in AT-II-treated HO-1<sup>+/-</sup> mice but rather further impaired vascular function and increased ROS formation in this setting (Figure 5E and F).

## **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 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 reactive oxygen species via activation of the vascular NADPH oxidase and xanthine oxidase contributed considerably to this phenomenon. Interestingly, in all models eNOS expression was up rather than down-regulated suggesting that eNOS might be dysfunctional, uncoupled under these circumstances<sup>4, 23-25</sup>.

Previously, it was conceptualized that treatment with an exogenous source of NO (e.g. 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, nitric oxide, rapidly reacts with superoxide to produce the highly reactive intermediate, peroxynitrite a potent oxidant, which 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 tetrahydrobiopterin (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 extra-cellular superoxide dismutase activity and lead 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 to upregulate the important antioxidant enzyme heme oxygenase I, which has been shown to play a key role in response to treatment with various organic nitrates, whether these animals develop tolerance and endothelial dysfunction<sup>21</sup>.

It remains to be established, however, whether PETN, a nitrate with antioxidant properties is able to improve endothelial dysfunction in an animal of endothelial dysfunction and oxidative stress. To address this issue we used the model of AT-II infusion where 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 NO synthase were identified.

Experiments with isolated mitochondria and membrane fractions from the heart revealed that PETN and not ISMN treatment was able to reduce significantly 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” radicals<sup>27</sup>. Thus all measures which 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 since it was recently demonstrated that GTN induced increases in NADPH oxidase-activity can trigger mitochondrial ROS formation via K<sub>ATP</sub> 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 two recent studies with diabetic rats we were able to demonstrate that the prevention of eNOS uncoupling in response to atorvastatin or the AT<sub>1</sub>-receptor blocker telmisartan was at least in part secondary to a upregulation of BH<sub>4</sub> synthesizing enzyme such as the GCH-I and 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 up rather than down-regulated in this animal model of hypertension. It is important to note that this is likely due 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 level<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 manuscripts 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, that 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, since 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 affirm with 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-enhanced chemiluminescence. The effects of PETN on vascular function in SHR were significantly different from those of ISMN, although less pronounced as compared to the effects in ATII-triggered hypertension. For detailed consideration of these differences see “Extended Results” in the online data supplement available at <http://hyper.ahajournals.org>. 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 anti-atherosclerotic 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 due 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 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.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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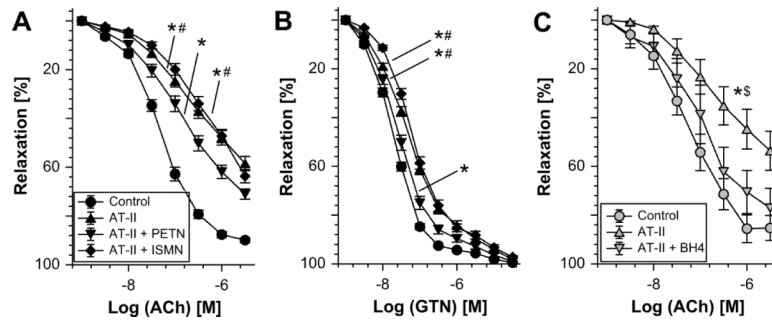
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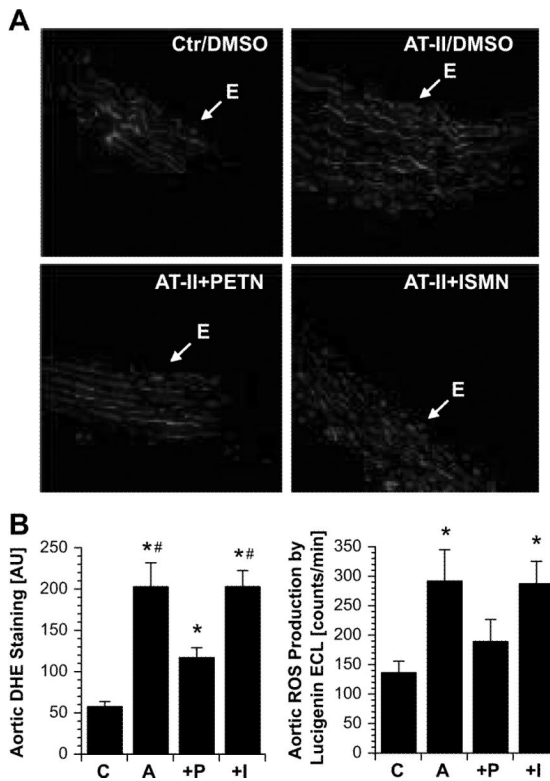
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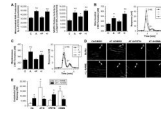
**Figure 1.**

Effects of in vivo PETN (15mg/kg/d) and ISMN (75mg/kg/d) treatment on the concentration response relationship to acetylcholine (ACh) (**A**) and nitroglycerin (GTN) (**B**) in aortic rings from angiotensin-II-infused (AT-II, 1mg/kg/d for 7d) rats. (**C**) The effect of sepiapterin (100  $\mu$ M), a BH<sub>4</sub> precursor, and PEG-SOD (100 U/ml) pretreatment of aortic rings from AT-II-infused rats for 1 h was determined in separate experiments. Data are the mean $\pm$ SEM of n=36–57 aorta from 10–15 rats per group (**A** and **B**) and n=6–8 from 3 rats per group (**C**). P < 0.05: \* vs. Ctr/DMSO; # vs. AT-II+PETN; \$ vs. AT-II/BH<sub>4</sub>. The statistics were based on 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 analysis (for sake of clarity significance is not shown for all data points).



**Figure 2.**

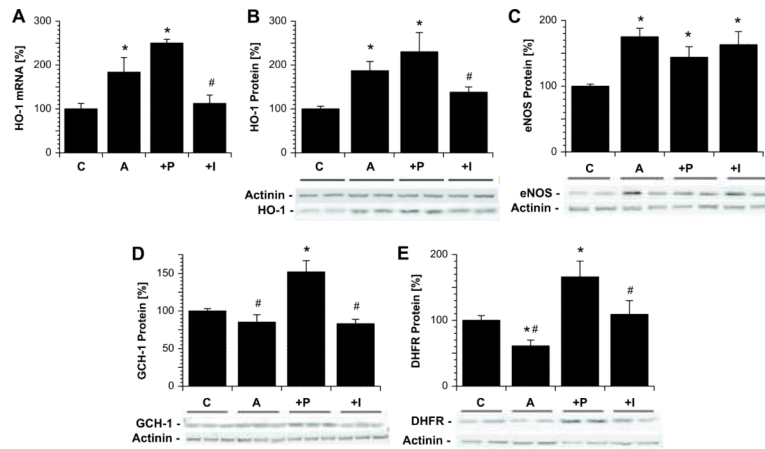
Effects of in vivo PETN and ISMN treatment on vascular superoxide levels (**A, B**) in angiotensin-II-infused (AT-II) rats. (**A**) Transverse aortic cryo-sections were labeled with dihydroethidine (DHE, 1 $\mu$ M), which produces red fluorescence when oxidized by reactive oxygen species. “E” indicates the endothelium; lamina autofluorescence is green. Pictures shown are representative for at least 6 animals/group. E means endothelium. (**B**) Densitometric quantification of the dihydroethidine-derived reactive oxygen species signal throughout the vessel wall (left panel) and lucigenin (5 $\mu$ M) enhanced chemiluminescence (ECL) in intact aortic ring segments (right panel). The data are mean $\pm$ SEM of n=18–19 experiments with tissue from at least 10 animals/group. P < 0.05: \* vs. Ctr/DMSO; # vs. AT-II+PETN. C, control; A, angiotensin-II-treated; P, angiotensin-II- and PETN-treated; I, angiotensin-II- and ISMN-treated.



**Figure 3.**

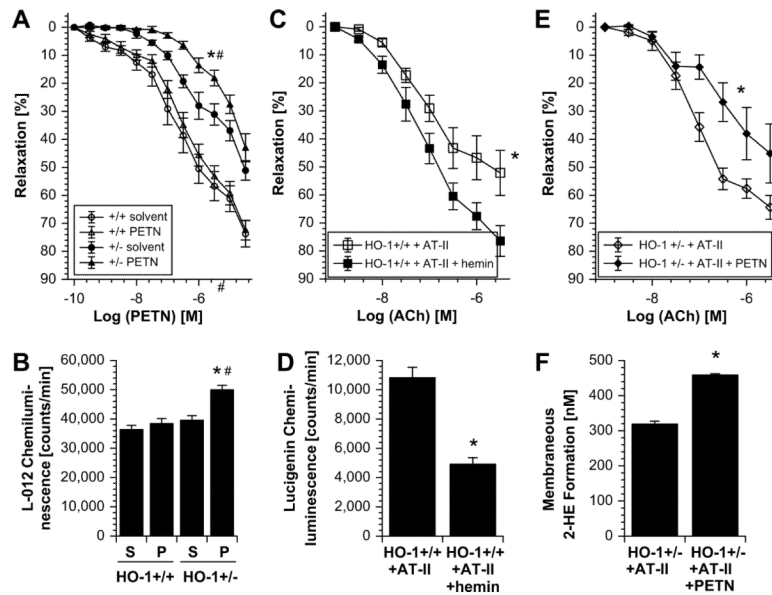
Effects of in vivo PETN and ISMN treatment on mitochondrial reactive oxygen species formation (**A, B**), NADPH oxidase activity (**A, C**) and eNOS-dependent reactive oxygen species formation (uncoupling) (**D, E**) in angiotensin-II-infused (AT-II) rats. (**A**) Reactive oxygen species formation in isolated cardiac mitochondria was measured by L-012 (100 $\mu$ M) enhanced chemiluminescence (ECL, left panel) and reactive oxygen species production (NADPH oxidase activity) in membrane fractions from hearts was determined by lucigenin (5 $\mu$ M) enhanced chemiluminescence (ECL, right panel). (**B, C**) Reactive oxygen species formation in isolated cardiac mitochondria and NADPH oxidase activity in membraneous fractions was also quantified by 2-hydroxyethidium levels. The inserts show representative HPLC chromatograms. E+ means ethidium, 2-HE means 2-hydroxyethidium. The data are mean $\pm$ SEM of n=31–43 (mitochondria and NADPH oxidase activity) experiments with tissue from at least 10 animals/group. The HPLC data are mean $\pm$ SEM of n=9 (mitochondria), n=15 (NADPH oxidase activity) experiments with tissue from 3–5 animals/group. (**D, E**) Fluorescence microscopy revealed reactive oxygen species formation by red staining (upper column). To determine eNOS-dependent reactive oxygen species formation, vessels were pre-incubated with the NOS inhibitor L-NAME (lower column). Densitometric data are presented by bar graphs (solid, w/o L-NAME and open, with L-NAME). Pictures and data shown are representative for at least 4 animals/group. For methodological details see “Figure S4” in the online data supplement available at <http://hyper.ahajournals.org>. P < 0.05: \* vs. Ctr/DMSO; # vs. AT-II+PETN. C, control; A, angiotensin-II-treated; P, angiotensin-II- and PETN-treated; I, angiotensin-II- and ISMN-treated.





**Figure 4.**

Effects of in vivo PETN and ISMN treatment on vascular heme oxygenase-1 (HO-1, mRNA and protein), eNOS, GTP-cyclohydrolase-I (GCH-I) and dihydrofolate reductase (DHFR) expression in aortic tissue from hypertensive rats. Expression of HO-1 mRNA (A) as well as HO-1 (B), eNOS (C), GCH-I (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 6–8 animals/group.  $P < 0.05$ : \* vs. Ctr/DMSO; # vs. AT-II+PETN. C, control; A, angiotensin-II-treated; P, angiotensin-II- and PETN-treated; I, angiotensin-II- and ISMN-treated.



**Figure 5.**

Effects of HO-1 deficiency versus HO-1 induction on vascular improvement by pentaerithryl tetranitrate (PETN). (**A**, **B**) PETN treatment (75mg/kg/d for 4d) 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, solvent; P, PETN-treated. (**C**, **D**) Hemin (25mg/kg i.p.)-triggered HO-1 induction improved high dose AT-II (1mg/kg/d for 7d)-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**, **F**) PETN (75mg/kg/d for 7d) failed to prevent endothelial dysfunction (ACh-response) induced by low dose AT-II (0.1mg/kg/d for 7d) in aorta from HO-1<sup>+/-</sup> mice. In accordance, PETN did not improve NADPH oxidase activity (2-HE formation by HPLC 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 data are mean  $\pm$  SEM of aortic rings and hearts from 4–5 animals/group.