Online Data Supplement

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

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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 described[6].

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 (0.5cm length) by lucigenin (5μM) ECL and dihydroethidine (1μM)-dependent fluorescence in aortic cryo-sections (fluorescent microtopography) as reported elsewhere[8]. 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 [8]. Mitochondrial ROS production and NADPH oxidase activity were determined by HPLC-based quantification of the conversion of dihydroethidine to 2-hydroxyethidium as described [9].

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 [10]. 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 [11, 12]. 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 BH_4/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 [13]. 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 [14]. 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 [15].

Extended References

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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.

	Potency (pD_2)§		Efficacy (%)	
In vivo Treatment	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.

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

* 80 mM KCl and 300 nM phenylephrine. n indicates the number of aortic rings per group.

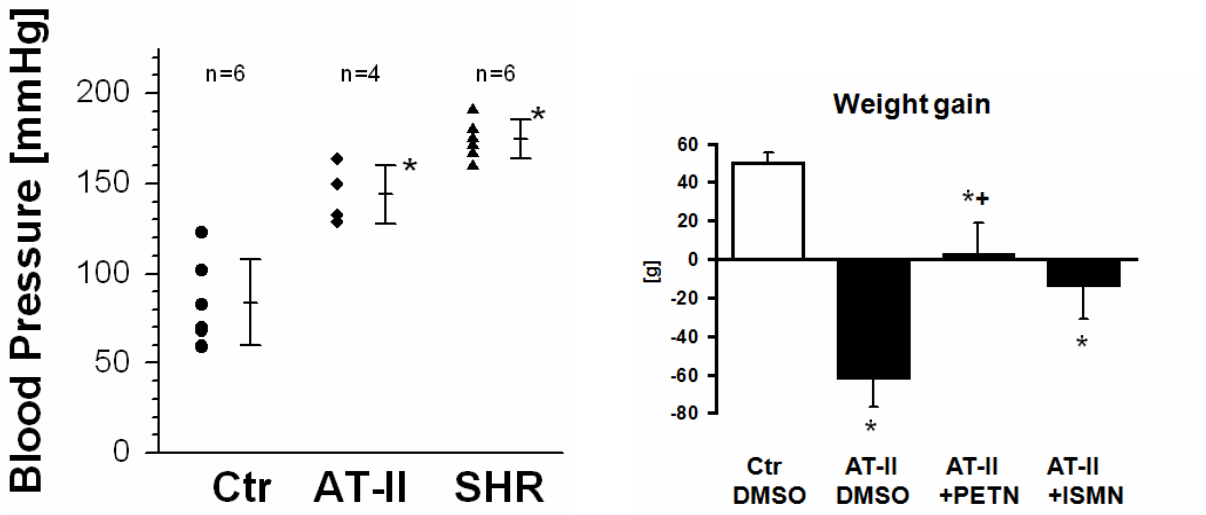

Figure S1. Left panel: Systolic blood pressure in control, AT-II-treated rats or SHR was measured by the tail cuff method. Data shown are the mean±SEM of at least 4 measurements. $P < 0.05$: * vs. Ctr. **Right panel:** Weight gain of rats in the different treatment groups (initial weight was 250g. Data shown are the mean±SEM of at least 8 measurements. $P < 0.05$: * vs. Ctr; $^{+}$ vs. AT-II.

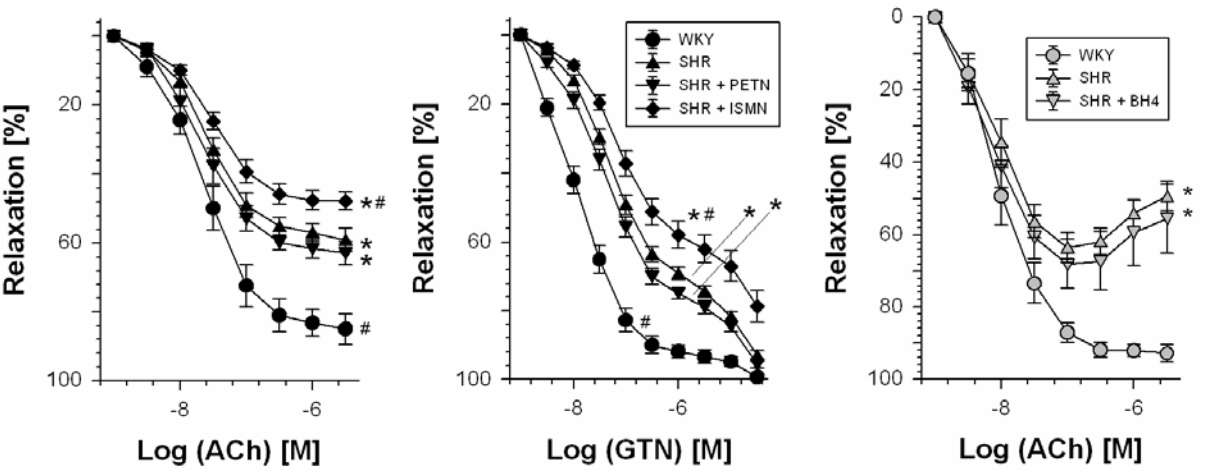

Figure S2. Effects of in vivo pentaerithrityl tetranitrate (PETN) and isosorbide-5-mononitrate (ISMN) treatment on the vasoreactivity of aorta from spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) controls. Endothelium-dependent (ACh, **A**) or –independent (GTN, **B**) vasodilation was assessed by isometric tension recording. (**C**) The effect of sepiapterin (100 µM), a BH_4 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 pD_2-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).

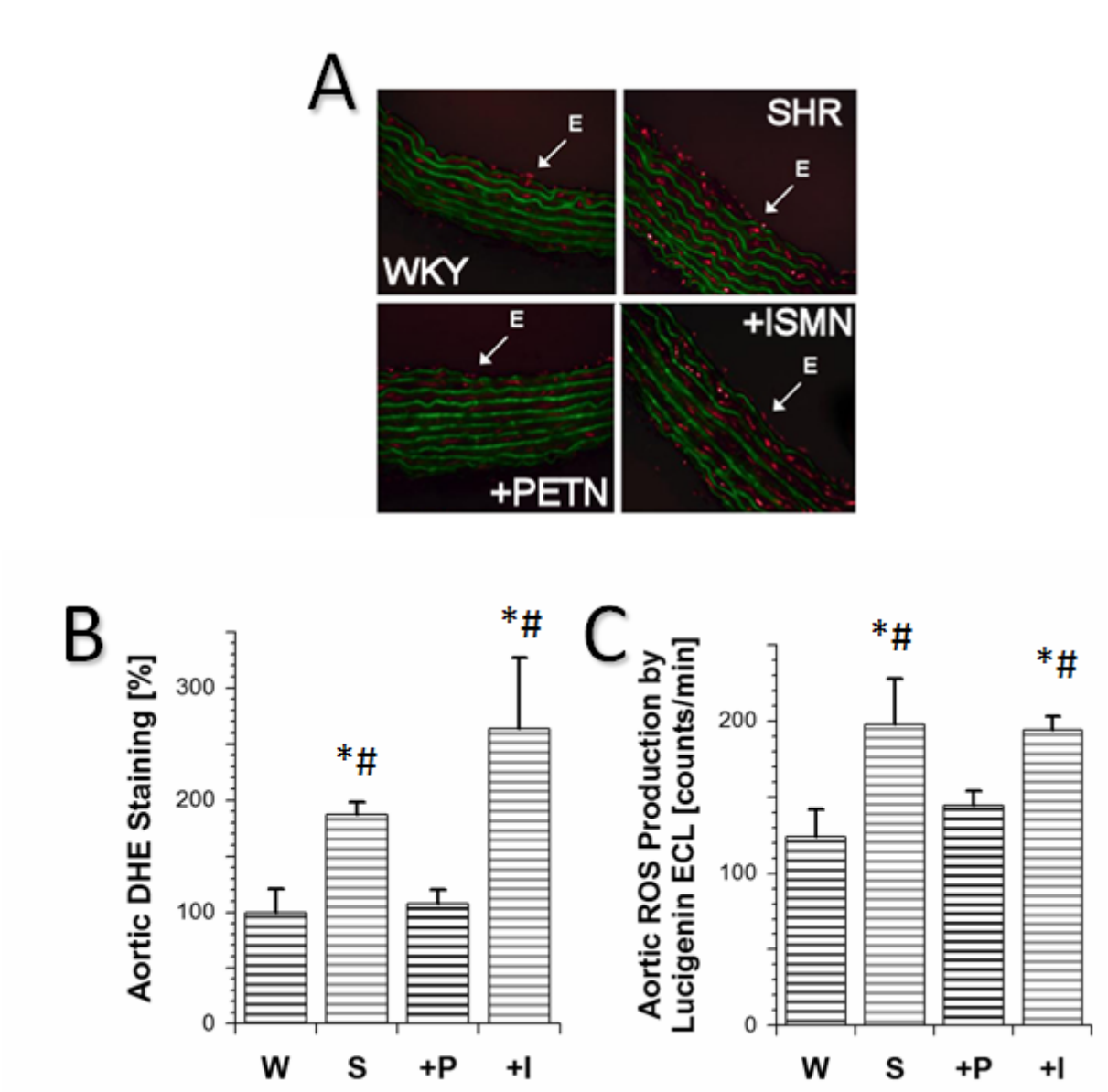

Figure S3. Effects of in vivo pentaerithrityl tetranitrate (PETN) and isosorbide-5-mononitrate (ISMN) treatment on the reactive oxygen species formation in aorta from spontaneously hypertensive rats (SHRs) and Wistar-Kyoto (WKY) controls. Dihydroethidine (DHE, 1µM) staining of aortic cryo-sections (**A**), 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.

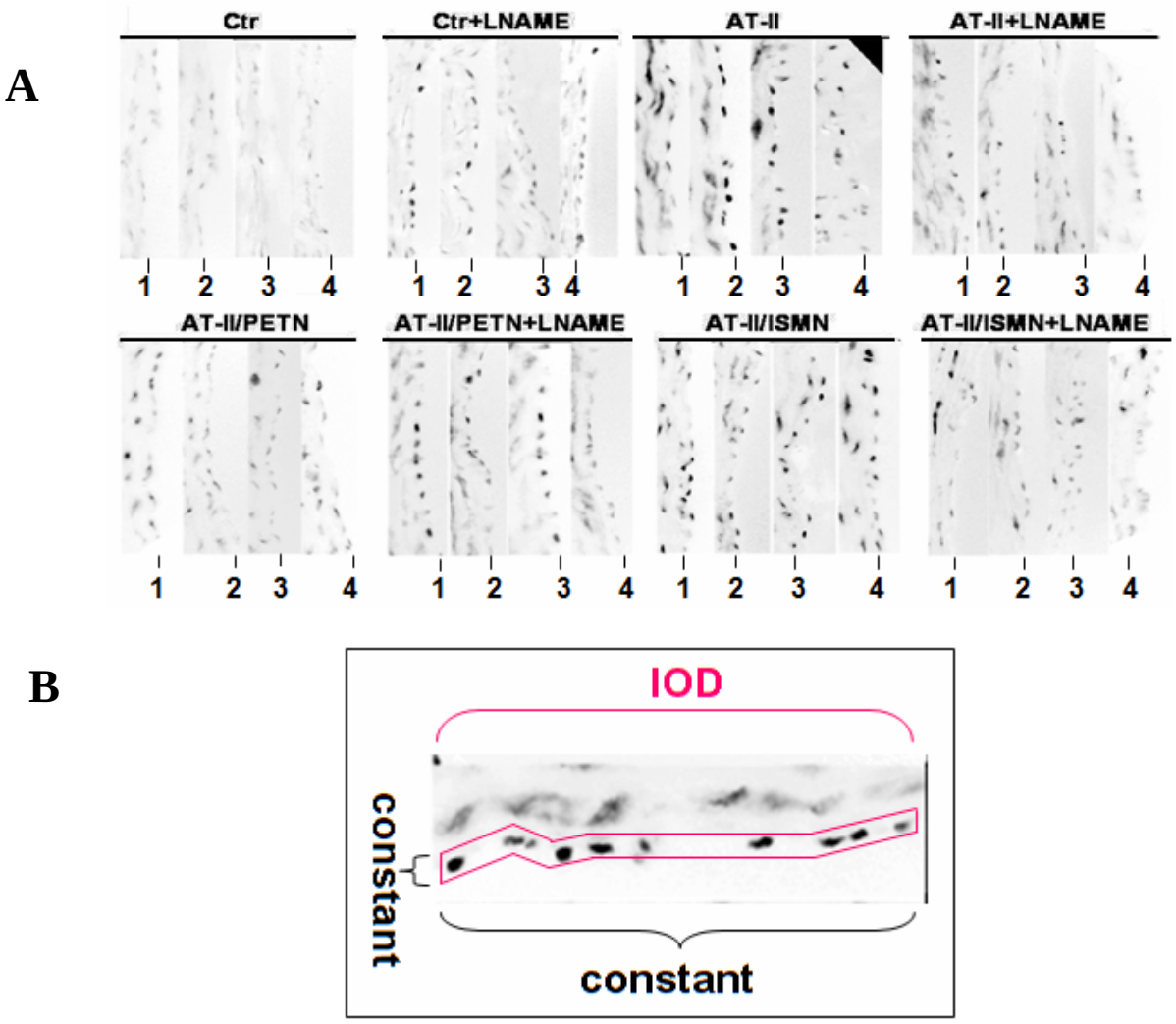

Figure S4. Effects of PETN tratment on eNOS uncoupling. Effects of in vivo pentaerithrityl tetranitrate (PETN) and isosorbide-5-mononitrate (ISMN) treatment on eNOS-dependent reactive oxygen species formation (uncoupling) in aorta from hypertensive rats. Dihydroethidine (DHE, 1µM)-fluorescent microtopography 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].