Fetal Programming

Maternal Treatment of Spontaneously Hypertensive Rats With Pentaerythritol Tetranitrate Reduces Blood Pressure in Female Offspring

Zhixiong Wu,* Daniel Siuda,* Ning Xia, Gisela Reifenberg, Andreas Daiber, Thomas Münzel, Ulrich Förstermann, Huige Li

See Editorial Commentary, pp 43–44

Abstract—Pentaerythritol tetranitrate is devoid of nitrate tolerance and shows no reproductive or developmental toxicity in animal studies. Recently, pentaerythritol tetranitrate has been demonstrated to reduce the risk of intrauterine growth restriction and the risk of preterm birth in women with abnormal placental perfusion. This study was conducted to test the perinatal programming effect of pentaerythritol tetranitrate in spontaneously hypertensive rats, a rat model of genetic hypertension. Parental spontaneously hypertensive rats were treated with pentaerythritol tetranitrate (50 mg/kg per day) during pregnancy and lactation periods; the offspring received standard chow without pentaerythritol tetranitrate after weaning. Maternal treatment with pentaerythritol tetranitrate had no effect on blood pressure in male offspring. In the female offspring, however, a persistent reduction in blood pressure was observed at 6 and 8 months. This long-lasting effect was accompanied by an upregulation of endothelial nitric oxide synthase, mitochondrial superoxide dismutase, glutathione peroxidase 1, and heme oxygenase 1 in the aorta of 8-month-old female offspring, which was likely to result from epigenetic changes (enhanced histone 3 lysine 27 acetylation and histone 3 lysine 4 trimethylation) and transcriptional activation (enhanced binding of DNA-directed RNA polymerase II to the transcription start site of the genes). In organ chamber experiments, the endothelium-dependent, nitric oxide–mediated vasodilation to acetylcholine was enhanced in aorta from female offspring of the pentaerythritol tetranitrate–treated parental spontaneously hypertensive rats. In conclusion, maternal pentaerythritol tetranitrate treatment leads to epigenetic modifications, gene expression changes, an improvement of endothelial function and a persistent blood pressure reduction in the female offspring. (Hypertension. 2015;65:232-237. DOI: 10.1161/HYPERTENSIONAHA.114.04416.) • Online Data Supplement

Key Words: epigenetics • fetal programming • hypertension • pentaerythritol tetranitrate

Hypertension is a major risk factor for stroke, myocardial infarction, heart failure, and end-stage renal disease. Currently, many antihypertensive therapies are available. Although generally effective and well tolerated by patients, these drugs cannot cure hypertension; a lifelong medication is necessary. Moreover, a significant number of patients are resistant to current antihypertensive treatments (hypertensive restriction and the risk of preterm birth in women with abnormal placental perfusion. This study was conducted to test the perinatal programming effect of pentaerythritol tetranitrate in spontaneously hypertensive rats, a rat model of genetic hypertension. Parental spontaneously hypertensive rats were treated with pentaerythritol tetranitrate (50 mg/kg per day) during pregnancy and lactation periods; the offspring received standard chow without pentaerythritol tetranitrate after weaning. Maternal treatment with pentaerythritol tetranitrate had no effect on blood pressure in male offspring. In the female offspring, however, a persistent reduction in blood pressure was observed at 6 and 8 months. This long-lasting effect was accompanied by an upregulation of endothelial nitric oxide synthase, mitochondrial superoxide dismutase, glutathione peroxidase 1, and heme oxygenase 1 in the aorta of 8-month-old female offspring, which was likely to result from epigenetic changes (enhanced histone 3 lysine 27 acetylation and histone 3 lysine 4 trimethylation) and transcriptional activation (enhanced binding of DNA-directed RNA polymerase II to the transcription start site of the genes). In organ chamber experiments, the endothelium-dependent, nitric oxide–mediated vasodilation to acetylcholine was enhanced in aorta from female offspring of the pentaerythritol tetranitrate–treated parental spontaneously hypertensive rats. In conclusion, maternal pentaerythritol tetranitrate treatment leads to epigenetic modifications, gene expression changes, an improvement of endothelial function and a persistent blood pressure reduction in the female offspring. (Hypertension. 2015;65:232-237. DOI: 10.1161/HYPERTENSIONAHA.114.04416.) • Online Data Supplement

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Hypertension is a major risk factor for stroke, myocardial infarction, heart failure, and end-stage renal disease. Currently, many antihypertensive therapies are available. Although generally effective and well tolerated by patients, these drugs cannot cure hypertension; a lifelong medication is necessary. Moreover, a significant number of patients are resistant to current antihypertensive treatments (hypertensive restriction and the risk of preterm birth in women with abnormal placental perfusion. This study was conducted to test the perinatal programming effect of pentaerythritol tetranitrate in spontaneously hypertensive rats, a rat model of genetic hypertension. Parental spontaneously hypertensive rats were treated with pentaerythritol tetranitrate (50 mg/kg per day) during pregnancy and lactation periods; the offspring received standard chow without pentaerythritol tetranitrate after weaning. Maternal treatment with pentaerythritol tetranitrate had no effect on blood pressure in male offspring. In the female offspring, however, a persistent reduction in blood pressure was observed at 6 and 8 months. This long-lasting effect was accompanied by an upregulation of endothelial nitric oxide synthase, mitochondrial superoxide dismutase, glutathione peroxidase 1, and heme oxygenase 1 in the aorta of 8-month-old female offspring, which was likely to result from epigenetic changes (enhanced histone 3 lysine 27 acetylation and histone 3 lysine 4 trimethylation) and transcriptional activation (enhanced binding of DNA-directed RNA polymerase II to the transcription start site of the genes). In organ chamber experiments, the endothelium-dependent, nitric oxide–mediated vasodilation to acetylcholine was enhanced in aorta from female offspring of the pentaerythritol tetranitrate–treated parental spontaneously hypertensive rats. In conclusion, maternal pentaerythritol tetranitrate treatment leads to epigenetic modifications, gene expression changes, an improvement of endothelial function and a persistent blood pressure reduction in the female offspring. (Hypertension. 2015;65:232-237. DOI: 10.1161/HYPERTENSIONAHA.114.04416.) • Online Data Supplement

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an epigenetic component to the pathogenesis in this genetic hypertension model. Despite some controversy, embryonic transfer or cross-fostering experiments between SHR and normotensive Wistar–Kyoto rats have largely demonstrated that both the prenatal and the postnatal environments have an effect on blood pressure development in SHR.5,6 Recent studies have identified some epigenetic mechanisms contributing to the high blood pressure in SHR.5,6

Therefore, it is conceivable that essential hypertension can be, at least partially, alleviated by epigenetic drug interventions. Jaap A. Joles and his colleagues have observed a long-lasting blood pressure reduction in SHR offspring by treatment of parental SHR during the last 2 weeks of gestation and until 4 or 8 weeks of age with either a combination of L-arginine plus vitamins C and E and taurine,7 with the nitric oxide (NO) donor molsidomine8 or with the L-arginine precursor L-citrulline.9 These effects may involve epigenetic mechanisms although epigenetic changes were not analyzed in those studies.

The studies of Joles’ group suggest that the blood pressure–lowering effects of the abovementioned perinatal interventions are largely attributable to antioxidative or NO-stimulating properties of the used compounds. Therefore, we chose to use pentaerythritol tetranitrate (PETN) in this study because this drug combines both antioxidative and NO-stimulating properties in a single molecule. PETN is the only drug among the organic nitrates, such as isosorbide-5-mononitrate or nitroglycerin.12–14 PETN not only releases NO like other organic nitrates but also additionally enhances endothelial NO production by preventing endothelial NO synthase (eNOS) uncoupling.12–14 Moreover, PETN also possesses multiple antioxidative effects, including induction of heme oxygenase 1 (HO-1)12 and superoxide dismutase 3 (SOD-3),13,15 inhibition of cardiac nicotinamide adenine dinucleotide phosphate oxidase and serum xanthine oxidase activity.13 These beneficial effects are not shared by other organic nitrates, such as isosorbide-5-mononitrate or nitroglycerin.12–14

By using PETN, we have found out in this study that blood pressure in the SHR female offspring can be persistently reduced by maternal treatment and the underlying mechanisms involve epigenetic modifications.

**Methods**

For detailed methodology, see the online-only Data Supplement. In brief, parental F0 SHR animals were treated with PETN (50 mg/kg per day) during pregnancy and lactation periods; the F1 offspring received standard chow without PETN after weaning. Vascular function, gene expression analyses (quantitative real-time reverse transcription polymerase chain reaction), histone 3 lysine 4 trimethylation (H3K4me3), histone 3 lysine 27 acetylation (H3K27ac), as well as binding of the DNA-directed RNA polymerase II polypeptide A to the proximal promoter region around the transcription start site of the interested genes (chromatin immunoprecipitation followed by quantitative polymerase chain reaction)16 were studied using aorta samples from female F1 offspring.

**Results**

**Maternal PETN Treatment Reduces Blood Pressure in the Female Offspring**

Before mating (at 3 months), F0 parental SHR in PETN and control groups had similar blood pressure (Table S2). The blood pressure was higher after weaning of the offspring than before mating, which represents a normal blood pressure development in these animals. Treatment throughout pregnancy and lactation with PETN had no effect on this blood pressure development in F0 animals, neither in male nor in female SHR (Table S2).

In F1 rats, we measured the blood pressure at 6 and 8 months. No difference in blood pressure was observed between PETN and control groups in male F1 offspring (Figure 1). Interestingly, treatment of F0 parental SHR during the breeding and lactation periods led to a clear blood pressure reduction in the female offspring (Figure 1). The systolic blood pressure of F1 female rats in PETN group was 13 and 10 mmHg lower than that of control group at the 6 and 8 months, respectively (Figure 1). Because the F1 rats had no contact with PETN from the time point of weaning (at the age of 3 weeks), the blood pressure reduction in the female F1 rats represents a long-lasting effect that persisted over a time period of 28 months.

Figure 1. Maternal pentaerythritol tetranitrate (PETN) treatment reduces blood pressure in the female offspring. Parental spontaneously hypertensive rats were treated with PETN (50 mg/kg per day) during pregnancy and lactation periods. Blood pressure was measured in the offspring at 6 and 8 months, respectively. DBP indicates diastolic blood pressure; MBP, mean blood pressure; SBP, systolic blood pressure. Columns represent means±SEM, n=8 (female PETN), 11 (female Control), 13 (male PETN), and 15 (male Control), respectively. *P<0.05, compared with Control.
Maternal PETN Treatment Regulates Vascular Redox Genes

The improved NO bioavailability may result, on one hand, from enhanced NO production by eNOS and, on the other hand, from reduced NO breakdown by superoxide. Therefore, we studied the expression of redox genes in the aorta of female F1 rats.

Nox proteins are the catalytic subunit of nicotinamide adenine dinucleotide phosphate oxidase, which represents the major sources of reactive oxygen species in the vasculature. In the aorta of 8-month-old female F1 rats, the mRNA expression of Nox1 was significantly downregulated in PETN group, whereas the mRNA expression of Nox2 and Nox4 was not changed by PETN treatment (Figure 3A).

Among the major antioxidant enzymes, a significant upregulation of mRNA expression could be observed for SOD2, glutathione peroxidase 1 (GPx1), and HO-1, whereas the mRNA expression of SOD1, SOD3, and catalase was not changed (Figure 3B and 3C) by PETN. The upregulation of SOD2, GPx1, and HO-1 could also be demonstrated at the protein level (Figure 4). Consistently, aortic superoxide levels were reduced in the PETN female offspring (Figure S4).

Maternal PETN Treatment Improves Endothelial Function

In aortas of the female F1 SHR, eNOS expression was significantly enhanced by maternal PETN treatment, both at mRNA (Figure 2A) and protein (Figure 2B) levels. In organ chamber experiments, the acetylcholine-induced vasodilation of the isolated aorta was clearly enhanced. Not only was the curve shifted to the left but also the maximal relaxation was increased by PETN (Figure 2C). The vasodilator response of SHR aorta to acetylcholine was completely endothelium-dependent because it was preventable by the NOS inhibitor N\(^\circ\)-nitro-L-arginine methyl ester (l-NAME; Figure 2C). The endothelium-independent relaxation to the NO donor sodium nitroprusside did not differ between aortas from control and PETN groups (Figure 2D). These results indicated that the enhanced vasodilation to acetylcholine was a result of improved endothelial NO bioavailability, which was supported by electron paramagnetic resonance experiments (Figure S4).

Maternal PETN Treatment Improves Endothelial Function

In aortas of the 8-month-old female F1 SHR, the mRNA expression of eNOS, SOD2, and HO-1 gene, H3K27 acetylation and H3K4 trimethylation were enhanced in the aorta from 8-month-old female PETN offspring (Figure 5). H3K27 acetylation and H3K4 trimethylation are active epigenetic marks occurring in transcriptional active euchromatin and usually associated with transcriptional activation. Consistently, the binding of RNA polymerase II polypeptide A to the transcription start site of eNOS, SOD2, and HO-1 was enhanced in the 8-month-old female F1 SHR of the PETN group (Figure 5). This is an indication of enhanced transcription because RNA polymerase II polypeptide A is the largest subunit of DNA-directed RNA polymerase II, the polymerase responsible for synthesizing mRNA in eukaryotes.

Discussion

In this study, we show that maternal treatment of SHR with PETN during the pregnancy and lactation periods leads to a persistent blood pressure reduction in the female offspring. This is likely to result from an improved endothelial function because of enhanced NO production by eNOS and reduced NO breakdown by superoxide. An upregulation of eNOS, SOD2, GPx1, and HO-1 was evident in the aorta of 8-month-old female offspring, which was associated with active epigenetic marks (H3K4 trimethylation).

Figure 2. Maternal pentaerythritol tetranitrate (PETN) treatment improves endothelial function in the female offspring. Parental spontaneously hypertensive rats were treated with PETN (50 mg/kg per day) during pregnancy and lactation periods. The expression of eNOS mRNA (A) and protein (B) in the aorta of 8-month-old female offspring was studied with quantitative real-time reverse transcription polymerase chain reaction and Western blot analyses, respectively. For functional studies, aortic rings were precontracted with norepinephrine and then relaxed with acetylcholine (C) or the NO donor sodium nitroprusside (SNP) (D), in the absence or presence of the NOS inhibitor 1\(^\circ\)-nitro-L-arginine methyl ester (l-NAME, 500 \(\mu\)mol/L). C+L, control+L-NAME; P+L, PETN+L-NAME. Data are presented as mean±SEM, n=8 to 11.

\(^*\)P<0.05, compared with Control (A and B) or PETN (C), eNOS indicates endothelial NO synthase; and NO, nitric oxide.
PETN is an organic nitrate currently in clinical use for angina pectoris. Importantly, PETN seems to be safe for the use in pregnancy. According to the international REPROTOX database (http://reprotox.org), PETN is not expected to increase the risk of congenital anomalies. No reproductive or developmental toxicity has been observed in rats treated with PETN (100, 500, or 1000 mg/kg per day) during the mating and pregnancy periods.18 Mice and rats receiving ≤10000 ppm PETN in feed (resulting in PETN doses of 600–3000 mg/kg per day) for 2 years showed no evidence of carcinogenicity or toxicity.19

In a recent prospective, randomized, placebo-controlled, double-blinded trial involving 111 pregnant women with abnormal placental perfusion at 19 to 24 weeks of gestation, PETN treatment (80 mg twice daily) decreased the risk of intrauterine growth restriction or perinatal death, as well as intrauterine growth restriction alone.20,21 Although there was no difference in the risk of developing preeclampsia among the groups, early onset preeclampsia was reduced in trend in the high-risk group. Patients receiving PETN had a significantly reduced risk for preterm birth <32 weeks in comparison with the placebo group.20,21 Collectively, PETN led to an improvement in pregnancy outcome in the intervention group. Except headache (n=1) or dizziness (n=1) in the PETN group, no further adverse events or side effects have been observed.21 The authors concluded that PETN is a feasible drug starting in midtrimester in pregnancies at risk to develop placental insufficiency, intrauterine growth restriction, and preterm birth.

We treated parental SHR with PETN and observed no effect on blood pressure in the F0 animals. This is consistent with previous studies. Treatment of SHR with PETN (200 mg/kg per day by gavage) for 6 weeks does not change blood pressure.22,23 Thus, the persistent blood pressure reduction in the female offspring is unrelated to the blood pressure of the parental animals. In other words, a blood pressure reduction in the parental animal is not necessary for the blood pressure–lowering effect in the offspring. We propose that the persistent blood pressure reduction in the female offspring may be attributed to following mechanisms such as (1) PETN treatment may lead to a change in the intrauterine and postnatal environments. Uteroplacental insufficiency in rats (induced by bilateral uterine artery ligation) is known to elevate blood pressure in the offspring. Therefore, it is conceivable that an improvement of uteroplacental blood flow may have an opposite effect, ie, blood pressure reduction in the offspring. Indeed, treatment of pregnant women with PETN reduces uteroplacental vascular resistance and improves uteroplacental perfusion20 and (2) during the pregnancy period, PETN may directly affect the fetus by crossing placenta. In the perinatal period until weaning, the pups may receive PETN through lactation18 and may access PETN directly. In both case, the blood pressure–lowering effect of PETN in the offspring must be a programming effect that occurs during the in-utero or perinatal periods because PETN has no direct effect on blood pressure.

Figure 3. Maternal pentaerythritol tetranitrate (PETN) treatment changes the mRNA expression of redox genes in the female offspring. Parental spontaneously hypertensive rats were treated with PETN (50 mg/kg per day) during pregnancy and lactation periods. Quantitative real-time reverse transcription polymerase chain reaction was performed using aortas from 8-month-old female offspring to study the mRNA expression of nicotinamide adenine dinucleotide phosphate oxidases (Nox1, 2, and 4), superoxide dismutases (SOD1–3), catalase, glutathione peroxidase 1 (GPx1), and heme oxygenase 1 (HO-1). Columns represent mean±SEM, n=8 to 11. *P<0.05, compared with control.

Figure 4. Maternal pentaerythritol tetranitrate (PETN) treatment changes the protein expression of redox genes in the female offspring. Parental spontaneously hypertensive rats were treated with PETN (50 mg/kg per day) during pregnancy and lactation periods. Western blot analyses were performed using aortas from 8-month-old female offspring to study the protein expression of SOD2, glutathione peroxidase 1 (GPx1), and heme oxygenase 1 (HO-1). Blots shown are representative for 11 Control samples and 8 PETN samples. Columns represent mean±SEM of densitometric analyses. *P<0.05, compared with Control.
hypertension to an average value of 1.6%. Thus, the aortic pressure–lowering effect of PETN in the female offspring was evident as late as at 8 months (later time points were not studied). Because of these long-lasting effects, we proposed that maternal PETN treatment also cause gene expression changes in the heart of the offspring. In optimal case, this gene regulation may lead to changes that resemble physiological cardiac hypertrophy. However, this notion remains speculative at this time point and needs to be verified in future studies.

The primary idea of this study was to combat genetic hypertension with epigenetic drug interventions. Currently, some histone deacetylase inhibitors (vorinostat and romidepsin) and DNA methyltransferase inhibitors (azacitidine and decitabine) are approved for the treatment of human cancer. Histone deacetylase inhibitors have also been shown to be beneficial in preclinical models of heart failure. However, the enzymes that catalyze DNA methylation or histone modifications do not per se confer any site-specificity. Targeting these enzymes will affect many epigenetic marks in a largely nonspecific manner, and thus may result in considerable adverse effects. On the basis of this consideration, we did not use such conventional epigenetic drugs in this study. We hypothesized that certain compounds may act as a kind of “atypical epigenetic drugs” by inducing epigenetic modifications without being direct inhibitors or activators of epigenetic enzymes (eg, histone deacetylase, DNA methyltransferase). Our data indicate the PETN represents such an example, when used during pregnancy and lactation periods.

In conclusion, maternal PETN treatment of SHR during pregnancy and lactation periods leads to a blood pressure reduction in the female offspring, which persists at least to 8 months. This blood pressure–lowering effect is associated with an enhanced expression of eNOS and antioxidant enzymes (HO-1, SOD2, and GPx1), a reduction of Nox1 expression and an improved endothelial function in the aorta. These changes are evident even at 8 months and are likely to be attributable to epigenetic modifications (H3K27 acetylation and H3K4 trimethylation) that occur during the in-utero and perinatal periods.

**Perspectives**

PETN is an organic nitrate currently in clinical use for angina pectoris. The drug is devoid of nitrate tolerance and shows no reproductive or developmental toxicity in animal studies. Recently, PETN has been shown to reduce the risk of intrauterine growth restriction or perinatal death, and the risk for preterm birth in women with abnormal placental perfusion. In this study, we demonstrate that maternal treatment of SHR (a rat model of genetic hypertension) during the pregnancy and lactation periods with PETN leads to a persistent blood pressure reduction in the female offspring. This perinatal programming effect of PETN may have clinical implications. PETN treatment during pregnancy may be beneficial for...
high-risk patients (eg, those with a family history of essential hypertension, with preeclampsia and abnormal placental perfusion), and, potentially also beneficial for their daughters. Admittedly, clinical evidence is still missing.

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Disclosures

PETN used in this study was provided by Actavis Deutschland, Langenfeld, Germany. A. Daiber and T. Münzel received honoraria and research grant support from Actavis Deutschland, Langenfeld, Germany.

References


Novelty and Significance

What Is New?

- We have uncovered a blood pressure-programming effect of pentaerythritol tetranytrate (PETN). Maternal PETN treatment of rats with essential hypertension leads to a persistent blood pressure reduction in the female offspring.

What Is Relevant?

- Because PETN shows no reproductive or developmental toxicity in animal studies and exerts beneficial effects in women with abnormal placental perfusion, PETN could be used in high-risk patients during pregnancy to alleviate hypertension of the children.

Summary

Genetic hypertension can be ameliorated by maternal/perinatal intervention. PETN may serve as an example of atypical epigenetic drugs for such treatments.
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Data Supplement

Maternal treatment of spontaneously hypertensive rats with PETN reduces blood pressure in female offspring

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Material and methods

Animals

Spontaneously hypertensive rats (SHR) were obtained from Charles River Laboratories (Sulzfeld, Germany). PETN (18% PETN with 82% D-lactose monohydrate) was provided by Actavis Deutschland GmbH, Langenfeld, Germany. PETN-lactose (5.5 g/kg; ≈ 1 g/kg PETN) was mixed into normal chow (ssniff GmbH, Soest, Germany) resulting in a dose of approximately 50 mg/kg/day (PETN). F0 parental SHR were fed with either normal chow (control) or PETN-containing chow ad libitum from the time point of mating (at the age of 3 month) to the end of lactation period. The F1 offspring rats from all groups received normal chow from the time point of weaning (at the age of 3 weeks). Totally 8 Control breeding pairs and 8 PETN breeding pairs were used. 8 female/13 male offspring from the PETN breeding pairs and 11 female/15 male offspring from the Control breeding pairs were included in the study. The animal experiment was approved by the responsible regulatory authority (Landesuntersuchungsamt Rheinland-Pfalz; 23 177-07/G 11-1-015) and was conducted in accordance with the German animal protection law and the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Blood pressure measurement

Systolic blood pressure, diastolic blood pressure, and mean blood pressure were measured noninvasively in conscious animals by using a computerized system (CODA Monitor, Kent Scientific) with a volume-pressure recording sensor and an occlusion tail-cuff [1, 2]. In a validation study by comparison to simultaneous radiotelemetry measurements in mice, the volume-pressure recording tail-cuff system has been demonstrated to provide accurate blood pressure measurements over the physiological range of blood pressure [1]. Animals were placed in individual holders. The occlusion cuff and the volume-pressure recording cuff were placed close to the base of the tail. After an adaptation period of 30 min on a warm pad, the tail cuff was inflated 5 times for acclimation before measurements were performed. The mean of a
minimum of 15 recordings on each occasion was taken. The measurements were performed at the same time of day by the same investigator as done in our previous studies [3-5].

Aorta isolation

The rats were killed with an overdose of pentobarbital. The thorax was opened, and the whole aorta was harvested. The aorta was placed immediately in oxygenated ice-cold Krebs-Henseleit solution gassed with a mixture of 95% O₂ and 5% CO₂. The aorta was cleaned of adhesive fat and connective tissue [6].

Vascular function studies

Thoracic aorta from 8-month-old female F1 rats was cut into rings of approximately 2 mm in length. The rings were mounted in an organ chamber and connected to a force transducer (Wire Myograph, Danish Myo Technology, Aarhus, Denmark). The organ chamber was filled with Krebs-Henseleit solution that was continuously oxygenated with carbogen and kept at 37°C (pH 7.4) [6, 7]. The rings were equilibrated for 60 minutes and contracted two times with 60 mM KCl. In vasoconstriction experiments, the rings were exposed to increasing concentrations of angiotensin II (Ang II), norepinephrine (NE) or endothelin 1 (ET-1) in a cumulative fashion. In vasodilation experiments, the rings were pre-contracted with NE to reach the submaximal tension (80% of that obtained with 60 mM KCl), before vasodilation was induced either by acetylcholine (ACh, endothelium-dependent) or by the NO donor sodium nitroprusside (SNP, endothelium-independent). In additional experiments, the rings were preincubated with the NOS inhibitor N⁶-nitro-L-arginine methyl ester (L-NAME, 500 μM) for 30 minutes, and the vascular response to NE and ACh performed in the presence of L-NAME.

Gene expression analyses

Isolated aortic samples were homogenized in liquid nitrogen. RNA was isolated from aorta samples using peqGOLD TriFast™ (30-2010, PEQLAB) according to manufacturer’s protocol. cDNA was generated with the High Capacity cDNA Reverse Transcription Kit (4368813, Applied Biosystems). Quantitative real time RT-PCR (qPCR) reactions were performed on a StepOnePlus™ Real-Time PCR System (Applied Biosystems) using SYBR® Green JumpStart™ Taq ReadyMix™ (Sigma-Aldrich) using 20 ng cDNA. Relative mRNA levels of target genes were quantified using comparative threshold C_T normalized to housekeeping gene Glycerinaldehyde-3-phosphate-Dehydrogenase (GAPDH). mRNA expression in control animals were set to 100%. Primers were designed as described previously [8] and the primer sequences are shown in supplemental Table S1.

Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation (ChIP) was performed using the SimpleChIP Enzymatic Chromatin IP Kit with magnetic beads (New England Biolabs/Cell Signaling Technology) according to the manufacturer’s instructions. In brief, isolated aortic samples were homogenized in liquid nitrogen and 20 mg aorta sample was used for ChIP experiments. Nuclear proteins were cross-linked to DNA with formaldehyde (final concentration 1%). After two wash steps with ice-
cold PBS+PMSF (phenylmethylsulfonylfluorid), cells were lysed in lysis buffer and DNA was fragmented using Micrococal Nuclease. The nuclear membrane was broken by sonication using a sonicator (Heat Systems/Qsonica, Newtown, Connecticut, USA). Three µg of DNA was used for each ChIP experiment and 200 ng DNA was used as input control. The DNA samples were incubated with the corresponding antibody overnight and then magnetic beads were added. Proteins were digested with proteinase K for 2 h at 65°C and then DNA was isolated using the provided spin columns. Finally, qPCR was performed to quantify the protein-DNA binding [8]. The primers for the ChIP-qPCR were: eNOS-forward CTG GCC CAC ACT CTT CAA GT, eNOS-reverse CCT AAG GAA AAG GCC AGG AC; SOD2-forward GCT GCT CTC CTC AGA ACA CG, SOD2-reverse AGC GCC TAG CTG TGT CCT T; HO-1-forward CAG AGT TTC CGC CTC CAA C and HO-1-reverse GTA GTC GCT TGC CTG TCG AG. The PCR products correspond to -50 to +34 (eNOS), -27 to +64 (SOD2) and +7 to +152 (HO-1) of the corresponding genes (positions relative to transcription start site, TSS). The antibodies used were: rabbit monoclonal antibody against H3K4me3 (17-614, Millipore), mouse monoclonal antibody against H3K27ac (17-683, Millipore), and rabbit polyclonal antibody against RNA polymerase 2a (POLR2A) (ab5131, Abcam).

**Western blot analyses**

Western blot analyses were performed with total protein samples (30 µg each) from rat aorta. The following primary antibodies were used: rabbit monoclonal antibody against β-tubulin I (T7816, Sigma-Aldrich), mouse monoclonal antibody against eNOS (610297, BD Transduction Laboratories), rabbit monoclonal antibody against GPx1 (3120, Epitomics), rabbit monoclonal antibody against HO-1 (2322, Epitomics) and rabbit polyclonal antibody against SOD2 (SOD-110, Enzo Life Sciences). Western blot was carried out as previously described [9, 10]. Protein samples were separated on a Bis-Tris gel and transferred to a nitrocellulose membrane. Blots were blocked in 5% milk powder in TBST (10 mM Tris-HCl, pH 7.4, 150 mM NaCl with 0.1% Tween 20) for one hour at room temperature. The primary antibodies were diluted in the same solution used for blocking at 4°C overnight. Blots were then washed in TBST and incubated with a horseradish peroxidase-conjugated secondary antibody diluted in 5% milk in TBST for one hour. After washing in TBST and then in TBS, the immunocomplexes were developed using an enhanced horseradish peroxidase/luminol chemiluminescence reagent (PerkinElmer Life and Analytical Sciences, Boston, MA) according to the manufacturer’s instructions. Densitometric analysis was performed using the Quantity One software (Bio-Rad).

**Electron paramagnetic resonance (EPR)**

EPR was used to measure NO and superoxide in aortas of the female offspring. For NO measurement, 5 aortic rings (each 2-mm in length) from female F1 offspring were incubated at 37°C for 30 min in Krebs solution in the presence of 1 µM A23187 and 200 µM colloid Fe(DETC)₂ as described previously [6]. EPR studies were performed on a table-top X-band spectrometer Miniscope MS400 (Magnettech, Berlin, Germany). Recordings were made at 77 K using a Dewar flask (Wilmad, Buena, New Jersey). Instrument settings were 10 mW of microwave power, 7000 mG amplitude modulation, 100 kHz of modulation frequency, 3319 G center field, 108 G sweep width, 60 s sweep time and 5 scans.
For detection of superoxide, the spin probe 1-hydroxy-3-carboxy-2,2,5,-tetramethyl-pyrrolidine hydrochloride (CPH, 1 mM) was used [11, 12]. Two samples were taken from the same aorta segment and paired measurements were performed, i.e. one in the absence and one in the presence of PEG-SOD (100 U/ml). The SOD-inhibitable portion of EPR signal was considered as superoxide production [11, 12].

Statistics

Results are expressed as mean±SEM (standard error of the mean). Student’s t test was used for comparison of PETN group with control group. P values < 0.05 were considered significantly different. For statistical analysis GraphPad Prism (GraphPad Software, La Jolla, CA, USA) was used.

References


Supplemental Table S1: qPCR primer sequences

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<th>Orientation</th>
<th>Sequence (5'→3')</th>
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<td>AACCTGGCAACCATGAACTC</td>
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<tr>
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<td>----------------</td>
<td>--------</td>
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<tr>
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<tr>
<td><strong>SBP (mmHg)</strong></td>
<td></td>
<td>168.9 ± 4.1</td>
</tr>
<tr>
<td>Before mating</td>
<td><strong>DBP (mmHg)</strong></td>
<td>127.2 ± 5.4</td>
</tr>
<tr>
<td></td>
<td><strong>MBP (mmHg)</strong></td>
<td>140.7 ± 4.9</td>
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<td><strong>SBP (mmHg)</strong></td>
<td>170.2 ± 10.1</td>
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<td><strong>DBP (mmHg)</strong></td>
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<tr>
<td></td>
<td><strong>MBP (mmHg)</strong></td>
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F0 animals were orally treated with PETN (50 mg/kg/day) from mating to weaning of the offspring. Blood pressure was measured with non-invasively by tail-cuff plethysmography. The experiment was performed in two series, with 3 Control and 3 PETN breeding pairs in the first series and 5 breeding pairs in each group in the second series. Blood pressure of F0 animals was measured in the second series of experiment. SBP, systolic blood pressure; DBP, diastolic blood pressure; MBP, mean blood pressure. Data are expressed as mean±SEM, n=5.
Supplemental Table S3:

Maternal treatment of SHR with PETN had no effect on bodyweight of the adult offspring.

<table>
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<td></td>
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<tr>
<td>6 months</td>
<td>208.4 ± 3.7</td>
<td>212.3 ± 2.2</td>
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<tr>
<td>8 months</td>
<td>216.4 ± 3.2</td>
<td>215.4 ± 3.5</td>
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<tr>
<td>n</td>
<td>11</td>
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Parental SHR were orally treated with PETN (50 mg/kg/day) from mating to weaning of the offspring. Bodyweight (g) of the offspring was measured at the age of 6 and 8 months, respectively. Data were expressed as mean±SEM. No statistical difference was found between Control and PETN groups.
Expanded Results

Maternal PETN treatment has little effect on the vasoconstrictor systems.

To find out whether the blood pressure reduction in the female offspring was a result of decreased vasoconstriction, we studied the major vasoconstrictor systems in the aorta from female F1 SHR.

The mRNA expression of angiotensin-converting enzymes ACE1 and ACE2, and that of angiotensin II receptors AT\(_1\) and AT\(_2\) was not changed by PETN treatment (supplemental Fig. S1A). In ex vivo experiments, the constrictor response to exogenous angiotensin II did not differ in aortas from control and PETN groups (supplemental Fig. S1B).

PETN treatment had differential effects on \(\alpha_1\) adrenoceptor subtypes in aortas of female offspring, with \(\alpha_{1B}\) being downregulated and \(\alpha_{1D}\) upregulated (supplemental Fig. S2A). However, the constrictor response of the aorta to exogenous norepinephrine was not changed by PETN in ex vivo experiments (supplemental Fig. S2B), indicating no net effect on \(\alpha_1\) receptor function.

**Figure S1. Effect of maternal PETN treatment on RAS components in F1 female rats.** Parental SHR were orally treated with PETN (50 mg/kg/day) from mating to weaning of the offspring. Aortas from female offspring were used for qRT-PCR experiments (A) or for vascular reactivity study (B). Data represent mean±SEM; n=8-11.
In the aorta of female offspring, the mRNA expression of endothelin receptor ET$_A$ was increased and ET$_B$ was not changed (supplemental Fig. S3A). In ex vivo experiments, a slightly increased constrictor response to exogenous endothelin 1 was observed in aorta from the PETN group. This increase, however, was relatively small and statistically not significant (supplemental Fig. S3B).

**Figure S2.** Effect of maternal PETN treatment on α1 adrenergic receptors in F1 female rats. Parental SHR were orally treated with PETN (50 mg/kg/day) from mating to weaning of the offspring. Aortas of female offspring were used for qRT-PCR experiments (A) or for vascular reactivity study (B). Data represent mean±SEM; n=8-11. *P<0.05, compared with Control.

**Figure S3.** Effect of maternal PETN treatment on endothelin receptors in F1 female rats. Parental SHR were orally treated with PETN (50 mg/kg/day) from mating to weaning of the offspring. Aortas of female offspring were used for qRT-PCR experiments (A) or for vascular reactivity study (B). Data represent mean±SEM; n=8-11. *P<0.05, compared with Control.
Parental SHR were orally treated with PETN (50 mg/kg/day) from mating to weaning of the offspring. Bioactive NO in the aorta of female offspring was assessed by electron paramagnetic resonance (EPR) spin trapping technique using colloid Fe(DETC)₂ (A). For superoxide measurement, the cyclic hydroxylamine CPH was used as spin probe (B). Paired experiments were performed, with and without PEG-SOD, respectively. Superoxide formation was assayed as SOD-inhibitable EPR signal. Data represent mean±SEM; n=4-7. *P<0.05, compared with Control.

Figure S5. Effect of maternal PETN treatment on renal gene expression in F1 female rats. Parental SHR were orally treated with PETN (50 mg/kg/day) from mating to weaning of the offspring. Kidney of female offspring were used for qRT-PCR experiments to study the mRNA expression of elastase 1 (Ela1, downregulated in SHR compared to normotensive WKY rats), epoxide hydrolase 2 (Ephx2, upregulated in SHR), acyl-CoA synthetase medium-chain family member 3 (Acsm3, upregulated in SHR), CD74 (downregulated in SHR) and glutathione S-transferase mu 1 (Gstm1, ownregulated in SHR). Shown values are ΔmRNA changes (PETN – Control). Data represent mean±SEM; n=4-6. *P<0.05, compared with Control.
Extended Discussion on renal mechanisms

We focused on vascular mechanisms in the present study. However, we don’t rule out a potential contribution of the kidney. In the past, several DNA microarray studies have been performed attempting to identify the hypertension candidate genes in the kidney of SHR [13-17]. Although the genes found by these studies are not completely identical, some genes are observed with high consistency, including elastase 1 (Ela1, downregulated in SHR compared to normotensive WKY rats), epoxide hydrolase 2 (Ephx2, upregulated in SHR), acyl-CoA synthetase medium-chain family member 3 (Acsm3, upregulated in SHR), CD74 (downregulated in SHR) and glutathione S-transferase mu 1 (Gstm1, downregulated in SHR). We therefore studied the effect of maternal PETN treatment on renal expression of the abovementioned genes. As shown in supplemental Fig. S5, maternal PETN treatment partially reversed these gene expression changes in SHR (i.e. upregulation of Ela1, downregulation of Ephx2 and Ascm3 by PETN). PETN had no significant effect on renal expression of CD74 or Gstm1 in the female offspring (Fig. S5).

Especially the upregulation of Ela1 and downregulation of Ephx2 by PETN are highly relevant. Ela1 is the gene with the largest decrease in expression in SHR kidney compared to WKY [17]. Extracellular matrix proteins are important for the regulation of elastic properties of the vascular system. It has been demonstrated that intraperitoneal injection of elastase (for 9 weeks) significantly lowers blood pressure in SHR [18]. Ephx2 has been shown to be the predominant gene upregulated in SHR kidney compared to WKY [17]. Epoxyeicosatrienoic acids (EETs) have potent vasodilatory effects in the circulation and play an important role in the regulation of renal blood flow, arterial resistance and systemic blood pressure. Ephx2 converts EETs to less active dihydroxyeicosatrienoic acids. Ephx2-knockout mice have lower blood pressure [19] and selective Ephx2 inhibitors reduce blood pressure in hypertensive mice [20, 21]. Therefore, upregulation of Ephx2 in SHR potentially contributes to hypertension due to enhanced EET hydrolysis and removal of vasodilatory eicosanoids. Conversely, downregulation of Ephx2 by maternal PETN treatment may be implicated in the blood pressure reduction in female offspring in our study.