Aus dem Institut für Pharmakologie
der Universitätsmedizin der Johannes Gutenberg-Universität

Effects of maternal PETN treatment of spontaneously hypertensive rats on blood pressure in the offspring

(Wirkung von maternaler PETN-Behandlung von spontan-hypertensiven Ratten auf den Blutdruck der Nachkommen)

D i s s e r t a t i o n

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vorgelegt von

Zhixiong Wu
aus Shanghai

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gewidmet meiner Familie

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List of abbreviations

11beta-HSD2	11 beta-hydroxysteroid dehydrogenase type 2
ACE-1	angiotensin converting enzyme-1
ACE-2	angiotensin converting enzyme-2
Ach	acetylcholine
Agtr1	angiotensin II receptor type 1
Agtr2	angiotensin II receptor type 2
Ang II	angiotensin II
APS	ammonium persulfate
Arnt	aryl hydrocarbon receptor nuclear translocator
BCA	bicinchoninic acid
BH_4	tetrahydrobiopterin
BP	blood pressure
BSA	bovine serum albumin
CAT	catalase
cDNA	complementary DNA
CO	carbon moNOXide
CTGF	connective tissue growth factor
CVD	cardiovascular disease
DGK	German Cardiac Society
DHFR	dihydrofolate reductase
DNA	Deoxyribonucleic acid
DNMT1	DNA methyltransferase 1
DNMTs	DNA methyltransferases
dsRNA	double-stranded RNA

EC-SOD	extracellular superoxide dismutase
EnaC-α	epithelial sodium channel-α
eNOS	endothelial nitric oxide synthase
ESC	European Society of Cardiology
ET-1	endothelin 1
ET_AR	endothelin receptor type A
ET_BR	endothelin receptor type B
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GCH-1	GTP cyclohydrolase 1
GPx1	glutathione peroxidase 1
GTP	guanosine-5'-triphosphate
HATs	histone acetyl transferases
HDACs	histone deacetylases
HMTs	histone methyltransferases
HO-1	heme oxygenase-1
HP1	heterochromatin protein 1
HRP	horseradish peroxidase
L-NAME	N^{ω}-nitro-L-arginine methyl ester
MECP-2	methyl CpG binding protein-2
miRNA	microRNA
Mn-SOD	manganese-SOD
mRNA	messenger RNA
NE	norepinephrine
NET	norepinephrine transporter
NF1	nuclear factor 1

NO	nitric oxide
PBMC	peripheral blood mononuclear cell
PETN	Pentaerithrityl tetranitrate
PETriN	pentaerythrityl trinitrate
PNMT	phenylethanolamine N-methyltransferase
qPCR	quantitative real-time polymerase chain reaction
RAS	renin-angiotensin system
RdRPs	RNA-directed RNA polymerases
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species
RT-PCR	reverse transcription polymerase chain reaction
RVLM	rostral ventrolateral medulla
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SERPINs	serine protease inhibitors
SHRs	Spontaneously hypertensive rats
SIRT1	sirtuin 1
snoRNA	small nucleolar RNA
SNP	sodium nitroprusside
snRNA	small nuclear RNA
SOD	superoxide dismutase
Tris-HCl	Tris(hydroxymethyl)aminomethane-HCl
VPR	Volume Pressure Recording

VSM	vascular smooth muscle
WKY	Wistar-Kyoto rat

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1 Introduction

Essential hypertension is defined as high blood pressure (BP) in which secondary causes such as renovascular disease, renal failure, pheochromocytoma, aldosteronism, or other causes of secondary hypertension or mendelian forms (monogenic) are not present (Carretero and Oparil, 2000). Essential hypertension accounts for 95% of all cases of hypertension (Carretero and Oparil, 2000). There are many genes that could participate in the development of essential hypertension (Kunes and Zicha, 2009). As a result of these complicate mechanisms, essential hypertension is a major risk factor for cardiovascular disease (CVD), such as stroke, myocardial infarction and heart failure. There is a strong positive and continuous correlation between BP and the risk of CVD and mortality (Carretero and Oparil, 2000).

Epigenetics refers to mechanisms for environment-gene interactions that do not alter the underlying base sequence of the gene (Millis, 2011). Recent researches found that epigenetics contributes to development of hypertension (Millis, 2011). DNA methylation and histone acetylation can play roles in epigenetics, by modifying the transcription of DNA into RNA. A lot of genes were found to be modified by epigenetic mechanisms in the blood vessel remodeling associated with hypertension (Millis, 2011).

Perinatal programming offers a new insight into the relationship between epigenetics and hypertension, which states that a nutritional insult during development will adapt to the immediate environment causing permanent alterations in tissue architecture, cell number and function, rendering the offspring metabolically disadvantaged at times of dietary fluctuations as an adult (Barker, 1995, Woodall et al., 1996, Gray et al., 2013). More recent studies in animal models have begun to characterize epigenetic modifications that are influenced by the intrauterine environment (Handy et al., 2011). Offspring from maternally undernourished dams showed a vascular dysfunction associated with hypertension (Torrens et al., 2009, Nuyt, 2008). Perinatal

programming of hypertension also inhibited nitric oxide (NO) synthase activity (Li et al., 2009), result in the reduction in NO (Franco Mdo et al., 2002, Hemmings et al., 2005, Nuyt and Alexander, 2009). Few studies have investigated perinatal interventions in offspring to reverse hypertension in later life (Nuyt and Alexander, 2009, Nuyt, 2008, Franco Mdo et al., 2003, Torrens et al., 2006).

Pentaerithrityl tetranitrate (PETN) is one organic nitrate used in the clinic. Compared to other organic nitrates, PETN is able to upregulate anti-oxidant enzymes thereby preventing nitrate tolerance and the development of endothelial dysfunction (Daiber et al., 2008). The upregulation of anti-oxidant enzyme heme oxygenase-1 (HO-1) might contribute to the protective effect of PETN on endothelial function.

Therefore, we tried to find the new role of PETN in perinatal programming and the possibility for treating essential hypertension. We used spontaneously hypertensive animal model to test whether perinatal PETN treatment could alleviate development of hypertension and restore the endothelial dysfunction in the offspring. Furthermore, we tried to explain the underlying molecular mechanism.

2 Literature discussion

2.1 Epigenetics

In 1942 Conrad Waddington coined the term epigenetics for "the branch of biology which studies the causal interactions between genes and their products which bring the phenotype into being" (Waddington, 2012). Today, epigenetics is generally defined as "the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence"(Wu and Morris, 2001). The Greek prefix *epi-* in *epigenetics* implies features that are "on top of" or "in addition to" genetics.

Epigenetic changes can switch genes on or off and determine which proteins are transcribed without altering the underlying DNA sequence. Additionally, the chromatin proteins associated with DNA may be activated or inhibited. This is why the differentiated cells in a multi-cellular organism express only the genes that are necessary for their own activity. An epigenetic system should be heritable, self-perpetuating, and reversible (Bonasio et al., 2010).

Specific epigenetic processes include paramutation, bookmarking, imprinting, gene silencing, X chromosome inactivation, position effect, reprogramming, maternal effects, the progress of carcinogenesis, many effects of teratogens, regulation of histone modifications and heterochromatin. For example, gene silencing can explain why genetic twins are not phenotypically identical. X chromosome inactivation makes one of the two copies of the X chromosome present in female mammals inactive (Egger et al., 2004).

Many factors and processes can affect epigenetics: development (in utero, childhood), environmental chemicals, drugs and pharmaceuticals, aging, and diet. Foods are known to alter the epigenetics of rats on different diets (Burdge et al., 2011). Three systems are involved in these epigenetic changes: they are DNA methylation, histone modification and RNA interference (Figure 1).

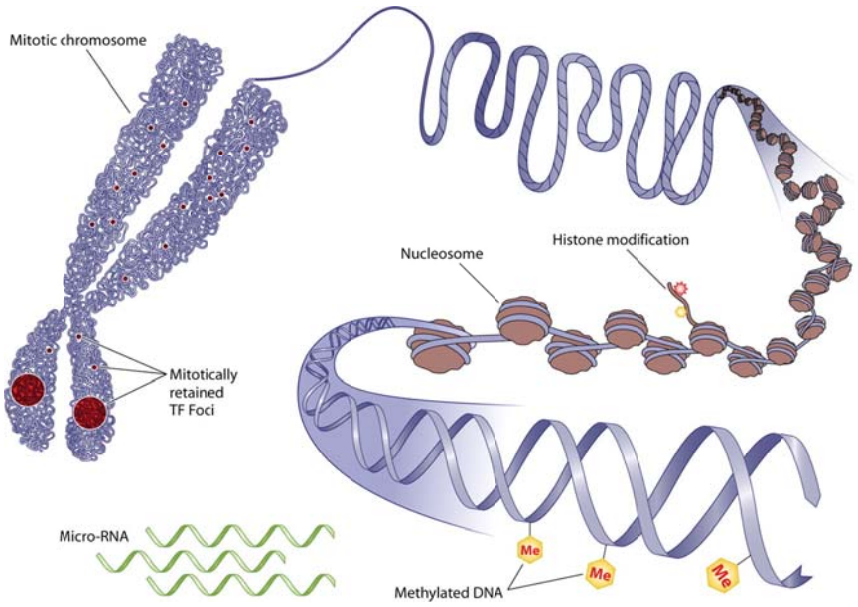

Figure 1: Mechanisms of inheritable epigenetics (Zaidi et al., 2010).

2.2 Epigenetics and hypertension

Recent studies indicate that epigenetic mechanisms contribute to the pathogenesis of essential hypertension.

Preeclampsia is the major pregnancy-induced hypertensive disorder. It modifies the expression profile of placental genes, including several serine protease inhibitors (SERPINs). Changed expression of SERPINA3, A5, A8, B2, B5, and B7 is found in pathological situations (Chelbi et al., 2007), associated with changed DNA methylation profiles. The DNA methylation level in the SERPINA3 gene is reported to be significantly decreased (hypomethylated) in placentas from pregnancies complicated by preeclampsia and fetal growth restriction, thereby providing potential markers for preeclampsia (Chelbi et al., 2007).

Another example addresses the role of chronic mental stress in the pathogenesis of hypertension. The reduced neuronal noradrenalin reuptake has an epigenetic mechanism. Phenylethanolamine N-methyltransferase (PNMT) is an enzyme that converts norepinephrine into epinephrine. PNMT induced by chronic mental stress in sympathetic nerve acts as a DNA methylase, which is involved in the mechanism of

binding of the methylation-related gene silencing transcription factor Methyl CpG binding protein-2 (MECP-2) to the DNA of the promotor-region, causing norepinephrine transporter (NET) gene silencing (Esler et al., 2008). Norepinephrine transporters are membrane proteins that conserve the catecholamine neurotransmitters norepinephrine and dopamine by transporting them back into the presynaptic neuron that released them. In essential hypertension, induction of PNMT by chronic mental stress leads to two consequences: first, epinephrine synthesis in sympathetic nerves; second, epigenetic silencing of NET gene leading to the phenotype of impairment of the neuronal re-uptake of norepinephrine by sympathetic nerves (Esler et al., 2008).

Aldosterone, the main regulator of sodium transport from activation of the renin-angiotensin system (RAS), is involved in an epigenetic pathway to control epithelial sodium channel-α (EnaC-α) gene expression in the collecting duct. Aldosterone disrupts the nuclear repressor complex, induces histone H3K79 hypomethylation and leads to activation of the ENaC-α promoter (Zhang et al., 2009). This epigenetic pathway is likely to contribute to renal fibrosis and genetic predilections for salt-sensitive hypertension (Lee et al., 2009).

The enzyme 11 beta-hydroxysteroid dehydrogenase type 2 (11beta-HSD2) is found to be relevant for blood pressure control and hypertension. Cortisol is degraded to cortisone by 11beta-HSD2. The mechanism that underlies the relationship between 11 beta HSD2 and hypertension is DNA methylation-induced hydroxysteroid dehydrogenase-11β2 (HSD11B2) gene repression, which encodes 11beta-HSD2 (Alikhani-Koopaei et al., 2004). Densely methylated CpG islands of the promoter and exon 1 of 11beta-HSD2 gene are correlated with low expression of this gene. In human peripheral blood mononuclear cell (PBMC), enhanced 11beta-HSD2 gene promoter methylation is associated with hypertension developing in glucocorticoid-treated patients. Patients of essential hypertension with elevated urinary tetrahydrocortisol- versus tetrahydrocortisone-metabolites (THFs/THE) ratio also show higher HSD11B2 promoter methylation (Friso et al., 2008). These results show a clear link between the epigenetic regulation through repression of HSD11B2,

by enhanced methylation of the CpG island in the promoter region, in the pathogenesis of essential hypertension.

2.3 Perinatal programming

The concept of perinatal programming was first mentioned as ‘fetal origins’ hypothesis, also referred to as the ‘Barker hypothesis’ (Barker, 1995). The hypothesis states that ‘alterations in fetal nutrition and endocrine status result in developmental adaptations that permanently change structure, physiology, and metabolism, thereby predisposing individuals to cardiovascular, metabolic, and endocrine disease in adult life (Barker, 1995). The observation was made in England, and led to the hypothesis that low birth weight babies who survived infancy and childhood might be at increased risk of coronary heart disease later in life. The relationship between reduced size at birth and diseases is described for diseases, such as hypertension, type II diabetes mellitus and hyperlipidemia (Barker, 1995).

Hypertension is a major risk factor for cardiovascular and cerebrovascular disease. Lifelong environmental factors (e.g., salt intake, obesity, alcohol consumption) and genetic factors clearly contribute to the development of hypertension. However, it has also been established that development in utero may program the later development of hypertension. One review examined 80 studies about the relationship of blood pressure with birth weight in children, adolescents and adults, and reported that blood pressure fell with increasing birth weight, the size of the effect being approximately 2 mmHg/kg (Huxley et al., 2000).

The link between fetal growth and adult hypertension must ultimately involve changes in gene expression, which are very likely to involve epigenetic phenomenon. More recent studies in animal models have begun to characterize epigenetic modifications that are influenced by the intrauterine environment (Handy et al., 2011).

2.3.1 Methyl donors diet

During early embryogenesis, DNA undergoes demethylation and remethylation; a process that involves 'labelling' of some genes as of maternal or paternal origin, and marks these genes for subsequent inactivation (Reik et al., 2001, de Boo and Harding, 2006). This epigenetic process of imprinting is thought to particularly affect many of the genes regulating fetal and placental growth (Reik et al., 2001). Methylation of DNA in the fetal liver is altered by low-protein diet during pregnancy in rats (Rees et al., 2000). In the mouse, methylation of DNA in the offspring has been shown to be altered by the level of methyl donors in the maternal diet (Wolff et al., 1998). Supplementation of a protein-restricted maternal diet in rats with methyl groups by the addition of folate or glycine has been shown to decrease hypertension (Jackson et al., 2002), improve endothelium-dependent vasodilation, increase eNOS mRNA levels (Torrens et al., 2006), and restore both the expression and promoter methylation status of the hepatic glucocorticoid receptor and peroxisome proliferator-activated receptor in offspring (Lillycrop et al., 2005). These researches support the hypothesis that folate and other methyl-group donors can influence fetal development and reduce the morbidity of hypertension in the next generation.

2.3.2 "Nephron underdosing"

Some evidence shows that perinatal programming is associated with hypertension by nephron number. Several researchers postulate that a low number of nephrons ('nephron underdosing') is a powerful predictor of hypertension later in adult life (Barker and Bagby, 2005, Brenner et al., 1988). This hypothesis has been confirmed by numerous animal studies (Cullen-McEwen et al., 2003, Horster et al., 1971, Wlodek et al., 2008).

2.3.3 Sympathetic activity

Sympathetic over activity may be also involved in the link between hypertension and low birth weight (Alexander et al., 2005). Reduction in sympathetic activity lowers blood pressure. Catecholamine is served as an indirect marker for sympathetic nerve

outflow, and an increased plasma concentration of catecholamine has been reported in numerous experimental models of perinatal programming (Hiraoka et al., 1991, Petry et al., 2000).

2.3.4 Vascular function

Vascular dysfunction is found in experimental animal models of programmed hypertension with exposure to low-protein diet, globally restricted diet, prenatal glucocorticoid exposure, and uterine insufficiency (Nuyt, 2008). In vivo, programmed hypertension is associated with unchanged or enhanced responses to vasoconstrictive agents such as angiotensin II, phenylephrine and endothelin 1, and is dependent on the vascular bed studied (conductance vs. resistance; cerebral vs. femoral vs. coronary arteries), the triggering insult, its timing and the age at which the offspring are studied. Vascular dysfunction is often amplified with aging and males seem more affected than females (Pladys et al., 2005, Nuyt and Alexander, 2009, Payne et al., 2003, McMullen et al., 2004, Roghair et al., 2005, Ozaki et al., 2001).

Endothelium-dependent vasodilatation is a well-recognized precursor of elevated blood pressure and atherosclerosis. Several studies demonstrate that endothelial-dependent and endothelial-independent vasodilatation can be impaired and that flow-mediated dilation is decreased in low birth weight individuals at birth, at 3 months of age, in later childhood and in early adult life (Nuyt, 2008, Franco et al., 2006, Singhal et al., 2004). Reduction in NO and prostaglandin levels participates in impaired vasodilatation (Franco Mdo et al., 2002, Hemmings et al., 2005). Vascular dysfunction can be associated with an enhanced production of superoxide anion and decreased expression of soluble guanylate cyclase (Nuyt and Alexander, 2009, Nuyt, 2008, Franco Mdo et al., 2003, Torrens et al., 2006). The mechanisms and cascade of events linking perinatal adverse conditions and adult vascular dysfunction are not completely revealed, and studies have not established so far whether vascular dysfunction and enhanced vascular generation of reactive oxygen species are primary or secondary to elevated blood pressure (Nuyt and Alexander, 2009).

2.3.5 Renin-angiotensin system (RAS)

The renin-angiotensin system (RAS) is a hormone system that regulates blood pressure and water balance. Studies have shown a role for RAS in perinatal programming, because treatment of pregnant mothers with angiotensin-converting enzyme inhibitors or angiotensin receptor antagonists alleviates hypertension in the offspring (Sherman and Langley-Evans, 1998). Furthermore, maternal protein deficiency during pregnancy also appears to substantially alter the fetal RAS. One study, using 3 days of water deprivation in pregnant rats, reported increments in fetal plasma sodium concentration and osmolarity in association with increments in fetal liver angiotensinogen mRNA and plasma angiotensin I and angiotensin II levels. Although there was no effect on basal blood pressures, blood pressures after angiotensin II administration were increased and baroreflex sensitivity was attenuated in the adolescent offspring of the water-deprived mothers. Heart angiotensin receptor mRNA and protein expressions were also higher in the water-deprived mothers and in their offspring (Guan et al., 2009).

In maternal low protein diet rat models of programming, the expression of the AT(1b) angiotensin receptor gene in the adrenal gland is upregulated, with the underlying mechanism of hypomethylation of the proximal promoter of the AT1b gene. In vitro, AT1b gene expression is highly dependent on promoter methylation (Bogdarina et al., 2007). When expression of the AT1b gene in the adrenal gland is upregulated by hypomethylation during the first week of life, increased expression of this receptor protein increases responsiveness of the adrenal gland to angiotensin II (Bogdarina et al., 2007). This scenario may contribute to an exaggerated response to salt-sensitive hypertension by epigenetic mechanisms.

Epigenetic regulation of brain RAS is important to programmed hypertension. Prenatal exposure to 50% low protein diet is reported to increase mRNA expression of angiotensinogen and angiotensin converting enzyme-1 (ACE-1), with a decrease in angiotensin II type 2 (AT2) receptor mRNA levels in mouse brain (Goyal et al., 2010). Angiotensinogen protein expression was unaltered, but ACE-1 and AT2 receptor

protein were decreased. These changes were associated with hypomethylation of the CpG islands in the promoter region of the ACE-1 gene and changes of various microRNAs, which regulate translation of ACE-1 and AT2, respectively (Goyal et al., 2010).

2.4 Pentaerythritol tetranitrate (PETN)

Pentaerithrityl tetranitrate (PETN) is one of organic nitrates used in the clinic. Organic nitrates represent a group of effective anti-ischemic drugs used for the treatment of patients with stable angina, acute myocardial infarction and chronic congestive heart failure. Compared to other organic nitrates, PETN is able to upregulate anti-oxidant enzymes thereby preventing nitrate tolerance and the development of endothelial dysfunction (Daiber et al., 2008).

Mitochondrial aldehyde dehydrogenase plays an important role for vasodilator potency of PETN. There appears to be a threshold of about 1-10 μM for PETN deciding over the pathway of bioactivation: below this threshold, the drug is predominantly metabolized by ALDH-2; above the threshold, unspecific bioactivation pathways involving enzymatic systems like P450s or reducing agents like low molecular weight thiols dominate (Figure 2) (Daiber et al., 2008). In vitro and in vivo studies suggest that PETN and its metabolite pentaerythrityl trinitrate (PETriN) induce the antioxidant defense protein heme oxygenase-1 (HO-1) which produces the antioxidant molecule bilirubin and the vasodilator carbon moNOXide (CO) by break-down of porphyrins (Oberle et al., 2003, Wenzel et al., 2007). HO-1 in turn stimulates the expression of a second antioxidant protein, ferritin, via the HO-1-dependent release of free iron from endogenous heme sources (Oberle et al., 1999). All together these defense mechanisms protected endothelial cells from hydrogen peroxide-induced toxicity, and might explain the observed antiatherogenic actions of PETN in vivo. As a result, PETN was observed to induce vasodilation in humans (Gori et al., 2003), and to reduce formation of atherosclerotic plaques in cholesterol-fed rabbits (Hacker et al., 2001).

Among the organic nitrates in clinical use, PETN is the only drug devoid of nitrate tolerance and endothelial dysfunction (Munzel et al., 2005). In contrast to nitroglycerin, PETN induces several cardioprotective genes and anti-oxidant enzymes. In addition, PETN reduces superoxide production from NADPH oxidase, xanthine oxidase and uncoupled eNOS (summarized (Li et al., 2013)).

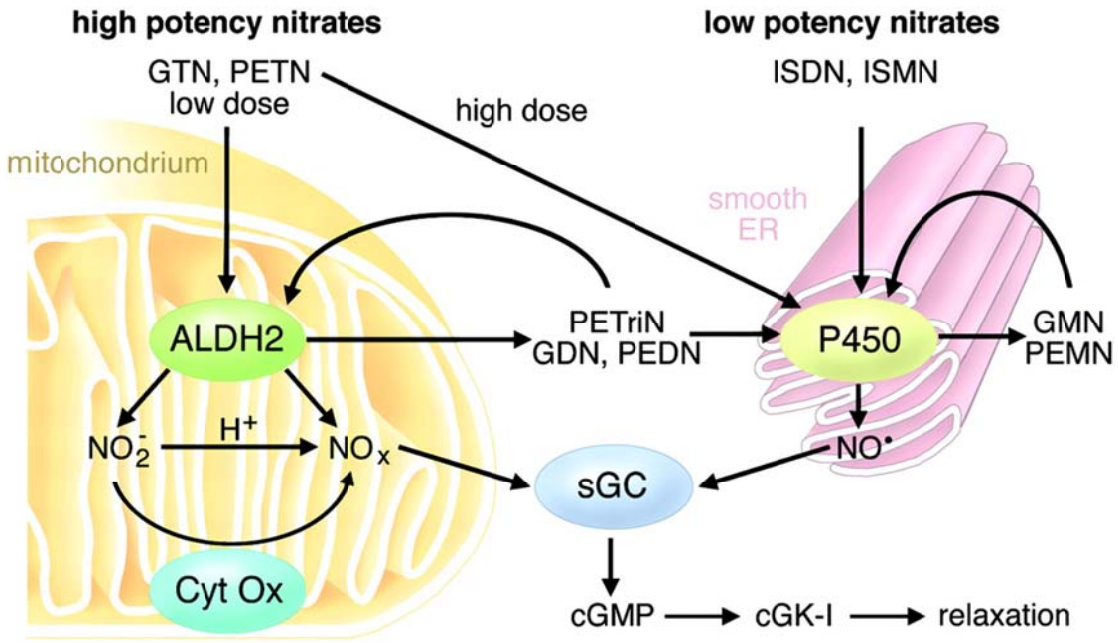

Figure 2: The scheme depicts proposed pathways of organic nitrate bioactivation in the vasculature (Daiber et al., 2008).

2.5 Aim of study

The aim of this study was to investigate:

- Whether maternal treatment of PETN can affect offspring's blood pressure and endothelial dysfunction?
- What are the molecular mechanisms?

3 Materials and methods

3.1 Spontaneously hypertensive rats

Spontaneously hypertensive rats (SHR) were obtained from Charles River Laboratories (Sulzfeld, Germany). The animal experiment was approved by the responsible regulatory authority (Landesuntersuchungsamt Rheinland-Pfalz; 23 177-07/G 11-1-015) and was performed in accordance with the German animal protection law and the guidelines for the use of experimental animals as stipulated by the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Experiment 1:

The F0 rats were 4 female spontaneously hypertensive rats and 4 male spontaneously hypertensive rats. They were maintained in standard cages in a room at 22±1 °C on a 12-h light cycle with 60% humidity. When they reached age of 3 months, the female rats were mated with male breeders. Pregnant rats were kept with male rats and randomly allocated into one of two groups: the control group (Control, n=2), fed with normal chow (V1536-000, ssniff GmbH, Soest, Germany) ad libitum; and the PETN group (PETN, n=2), fed with PETN-containing chow ad libitum. PETN was provided by Actavis Deutschland GmbH, and was mixed into standard chow at a concentration of 1 g/kg by ssniff GmbH, resulting in a dose of approximately 50 mg/kg/day. The animals were allowed free access to drinking water. All of the F0 rats were able to complete the gestation period. All of the F0 female rats delivered the F1 generation by spontaneous vaginal delivery. The PETN treatment continued until weaning. The lactation period was about 3 weeks. The rats were accompanied by parents until weaning. Then, the offspring were divided by gender and placed into separate cages. After separation from their parents, all the offspring received standard chow diet ad libitum. Body weight, blood pressure and heart rate of the F1 offspring were measured at the age of 6 and 8 months, respectively.

Experiment 2:

The procedure was almost the same as the first experiment, which is described above. F0 control group consisted of 5 pairs of SHR, and F0 PETN group 5 SHR pairs. The blood pressures and heart rates of F0 rats were measured before breeding and after weaning. The body weights and blood pressures of F1 rats were measured at the age of 3, 4 and 5 months, respectively.

3.2 Blood pressure measurement

Systolic blood pressure (BP), diastolic blood pressure, mean blood pressure and heart rate were measured in conscious and slightly restrained rats by tail-cuff volume pressure recording sensor (CODA Monitor, Kent Scientific, Torrington, USA).

The CODA system utilizes Volume Pressure Recording (VPR) sensor technology. The non-invasive BP methodology consists of utilizing a tail cuff placed around the tail to occlude blood flow and a tail cuff incorporating the VPR sensor placed distal to the occlusion cuff to measure BP parameters (Malkoff, 2005). When the occlusion cuff is slowly deflated, the VPR cuff measures the physiological characteristics of the returning blood systolic blood flow resulting in values for systolic and diastolic BP, mean BP, heart rate, tail blood volume and tail blood flow (Malkoff, 2005). A proper size rodent restrainer is essential for accurate BP measurements. Restrainers must comfortably restrain the animal while not creating stress and/or impeding normal posture and relaxed breathing (Malkoff, 2005). A darkened nose cone is beneficial in reducing the animal's view and reducing stress.

Appropriate animal preparation is necessary for accurate BP measurement. The rats were allowed to enter the restrainer freely; the restrainer was adjusted to hold the animal comfortably. The nose cone was adjusted to hold the rats comfortably while not allowing excessive movement, such as turning around.

Proper environmental temperature is essential for accurate blood pressure measurements (Malkoff, 2005). If the room is too cool, tail blood flow will be reduced. Our measurement was performed in a room at 22±1 °C.

Core body temperature is important for accurate and consistent BP measurement. The animal must have adequate blood flow to the tail for accurate measurement (Malkoff, 2005). The animal should be kept warm and comfortable but never hot. Overheating will elevate respiration rate and induce stress that can result in inconsistent and inaccurate BP readings (Malkoff, 2005). The heating pad was set at 80% heating and pre-heated for 30 minutes to reach about 32 °C. Then, the holders with the conscious rats were putted on the pad for 30 minutes. The animal's core body temperature was carefully managed. The heating period of 30 minutes is long enough to gain the tail signaling without over-heating.

The measurements were performed from 13:00 to 18:00 by the same investigator. After pre-heating, the CODA machine was connected to the computer, and the CODA software (Kent Scientific, Torrington, CT) was started. The device manager in the software automatically opened and searched for attached CODA device. After heating the rats for 30 minutes, the measurement could be started. An appropriate-sized occlusion cuff was slided up as near as the base of the tail without force. Forcing the cuff will cause occlusion of vessels resulting in poor BP measurement. The VPR cuff was slided up the tail with the larger end first, until reaching the occlusion cuff without force. The blood pressure and heart rate were determined in 15 cycles for each animal. Data was displayed and saved as a CSV spreadsheet for review.

3.3 Aorta Isolation

The rats were anesthetized with isoflurane, the thorax was opened, and the whole aorta was harvested. The aorta was placed immediately in oxygenated ice-cold Krebs-Henseleit solution (Table 1) gassed with carbogen (a mixture of 95% O_2 and 5%

CO_2). The aorta was cleaned of adhesive fat and connective tissue. Care was taken not to stretch the vessel and not to damage the endothelium or smooth muscle cells.

3.4 Vascular Reactivity Studies

In the second experiment, the thoracic aorta from 4-month-old rats was cut into rings approximately 2 mm in length (Watts, 2002). The remains were shock frozen in liquid nitrogen, and then stored at -80°C for further experiments.

Table 1: Krebs-Henseleit solution components

	MW	Supplier	Concentration (mM)
NaCl	58.44	3957, Carl Roth, Karlsruhe, Germany	118
$NaHCO_3$	84.01	6329, Merck KGaA, Darmstadt, Germany	25
KCl	74.56	6781, Carl Roth, Karlsruhe, Germany	4.8
$MgSO_4 \cdot 7H_2O$	246.48	5886, Merck KGaA, Darmstadt, Germany	1.2
KH_2PO_4	136.09	3904, Carl Roth, Karlsruhe, Germany	1.2
$Na_2EDTA \cdot 2H_2O$	372.24	A3553, AppliChem, Darmstadt, Germany	0.026
$Glucose \cdot H_2O$	198.17	6780, Carl Roth, Karlsruhe, Germany	11.1
$CaCl_2 \cdot 2H_2O$	147.02	2382, Merck KGaA, Darmstadt, Germany	2.5

Each aortic ring was mounted between 2 stainless-steel wires in a 10-ml organ bath and connected to a force transducer (Wire Myograph, DMT 610M and DMT 620M, Danish Myo Technology, Aarhus, Denmark). One wire was connected to a force transducer; the other wire was fixed at the lift arm of the Myograph. The organ bath was filled with Krebs-Henseleit solution (Table 1: Krebs-Henseleit solution), that was continuously oxygenated with carbogen and kept at 37°C (pH 7.4). The rings were placed under an optimal resting tension of 10 mN. Isometric tension was recorded by using an isometric force displacement transducer connected to a data acquisition system (PowerLab 8/S, AD Instruments Pty Ltd, Castle Hill, Australia). The software PowerLab Chart 5 (ADInstruments) was used for data acquisition and display. The rings were equilibrated for 60 minutes until a stable resting tension was acquired. The Krebs-Henseleit solution was changed every 15 minutes. Each ring was contracted two times with 60 mM KCl in order to establish a reproducible contractile response.

Thereafter, rings were rinsed with Krebs-Henseleit solution three times for 30 minutes, until baseline tone was restored. Endothelium integrity was confirmed by a relaxant response to 10^{-6} M acetylcholine after pre-constriction to 10^{-6} M norepinephrine.

The rings were exposed to increasing doses of norepinephrine (NE, 10^{-9} to 10^{-7} M), until the tension reached submaximal tone (about 80% of the steady-state tension obtained with isotonic external 60 mM KCl). Cumulative concentration of NE was expressed in half log increments. Responses were recorded in mN. In order to detect the endothelium-dependent dilation, acetylcholine (Ach) was introduced into this experiment. Sodium nitroprusside (SNP), an NO donor, was used to induce endothelium-independent vasodilation. Cumulative concentrations of Ach and SNP were expressed in half log increments. Responses were expressed as percentage relaxation of NE-induced tone, and relaxation in the absence of drugs (Ach or SNP) was set 0%. The contraction-relaxation experiment repeated twice. Between every repeat, the aortic ring was washed with Krebs-Henseleit solution three times for at least 30 minutes, until baseline tone was restored.

In order to detect the vascular reactivity to angiotensin II or endothelin 1, the rings were exposed to angiotensin II (Ang II, 10^{-9} to 10^{-7} M) or endothelin 1 (ET-1, 10^{-9} to 10^{-8} M) in a cumulative fashion. Responses were recorded in mN. Cumulative concentrations of Ang II and ET-1 were expressed in half log increments.

N^{ω}-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NO synthases, was added to the solution 30 minutes before NE to observe the effects on acetylcholine-induced relaxation. The final concentration of L-NAME is 500 μM. The rings were exposed to increasing doses of NE (10^{-9} to 10^{-8} M), until the tension reached submaximal tone (about 80% of the steady-state tension obtained with isotonic external 60 mM KCl). Then, Ach (10^{-9} to 10^{-6} M) was added in a cumulative fashion. The constriction was expressed as tension mN, and the relaxation was expressed as percentage of the max constriction induced by NE, and relaxation in the absence of Ach was set 0%.

3.5 Nucleic acid isolation

3.5.1 Phenol-chloroform extraction (TriFast™)

The phenol-chloroform extraction method serves to isolate nucleic acids. The nucleic acids are collected and divided from proteins by phase separation. Phenol dissolves proteins and lipids and leaves water soluble matters, like carbohydrates and nucleic acids, in the aqueous phase. Because phenol is denser, the organic phase is at the bottom of the tube, and the aqueous phase sits on the organic phase. Between these two phases an interphase can be seen. Because phenol is less polar than water, and nucleic acids are polar due to their negatively charged phosphate backbone, nucleic acids are soluble in water but not in phenol. When water and phenol are mixed, the nucleic acids remain in the aqueous phase, because polar molecules are best solved in polar solvents and non-polar molecules dissolve better in non-polar solvents. Chloroform is added to make sure that the phenol and water will mix again. At last, RNA is in the aqueous phase, DNA in the interphase and proteins are in the organic phase. Proteins have different side chains with different polarity. In the cytoplasm the proteins are mainly folded in the way that the amino acids with the polar side chains are on the outside and the hydrophobic side chains are away from the polar solvent, in case of the cytoplasm water. When proteins come in contact with a less polar solvent than water, in this case phenol, the non-polar side chains from the amino acids in the inside of the molecule want to get in contact with the new solvent, whereas the hydrophilic amino acids want to avoid the contact with the phenol. In phenol proteins are denatured and that is the reason while proteins and nucleic acids can be separated by phenol (Chomczynski and Sacchi, 2006).

In our study, 20 mg rat aorta was homogenized in liquid nitrogen with mortar. After homogenization, the sample powder was collected into a 1.5 ml tube filled with 500 µl TriFast™(peqlab, Erlangen, Germany). It is essential to use enough TriFast™ reagent, because an insufficient amount may cause contamination of RNA with DNA. The TriFast™ reagent contains phenol and guanidine isothiocyanat. Phenol lowers the pH and dissolve the proteins, whereas guanidine isothiocyanat lyses the cells and

denatures proteins, because it is a chaotropic salt. After tissue homogenization and lysis with the 500 µl of TriFast™ reagent, an incubation time of five minutes follows to ensure the dissociation of the nucleotide complexes. After the incubation, 100 µl of chloroform (A1585, Applichem, Darmstadt, Germany) was added into Trifast™. The samples were shaken fiercely and then incubated for 5 minutes at room temperature.

Then, the samples were centrifuged for 5 minutes at 13,000 x g. In the next step, the 200 µl of the aqueous phase, i.e. upper phase, are transferred to a new sample tube and 250 µl of isopropanol (6752, Carl Roth, Karlsruhe, Germany) was added. To support the precipitation of the RNA, 1 µl GlycoBlue™ (AM9515, Ambion/Life Technologies, Carlsbad, California, USA) was added to each sample. GlycoBlue™ is a blue dye covalently linked to glycogen and serves as a coprecipitant or carrier for nucleic acids. A useful side effect is that GlycoBlue™ labels the pellet blue, so it is easier seen in the sample tubes. The samples were vortexed thoroughly for about 15 seconds and then placed at -20°C for 10 minutes. After the precipitation, a centrifugation step (at 4°C for 20 minutes at 13,000 x g) followed. The RNA pellet could now be seen as a small dot at the bottom of the tube. The isopropanol supernatant was discarded carefully and the RNA pellet was washed twice with 500 µl 75 % ethanol at 4°C for 5 minutes at 13,000 x g. After each washing step the supernatant was discarded and the samples was placed head down to dry for 30 minutes under a chemical hood. Finally the dried RNA pellets were resuspended in 100 µl RNase-free water.

3.5.2 Concentration measurement of nucleic acids (NanoDrop™)

To determine the concentration of the isolated nucleic acids by the methods described above (2.4) the NanoDrop™ 1000 (Thermo Scientific, Wilmington, DE, USA) was used. All nucleotides absorb light at a maximum wavelength of 260 nm; this is due to nitrogen atoms in the purines and pyrimidines. That is the reason why the concentration of nucleic acids can be determined spectrophotometrically. The mathematical principles (from the NanoDrop™ 1000 manual) are a modified Lambert-Beer equation for nucleic acids to give $c = (A * \varepsilon)/b$. Where c is the nucleic

acid concentration in ng/ml, A is the absorbance in absorbance unit (AU), ε is the wavelength-dependent extinction coefficient in ng-cm/ml and b is the path length in cm. The generally accepted extinction coefficients for nucleic acids are:

• Double-stranded DNA: 50
• Single-stranded DNA: 33
• RNA: 40

Contaminants like proteins or phenol absorb light at 280 nm, because of their aromatic side chains. To determine the purity of nucleic acids the ratio of absorbance at 260 nm and 280 nm is used. A ratio of 1.8 is accepted as pure DNA, whereas a 260/280 ratio of 1.6-2.0 is accepted for RNA. The NanoDrop™ was first blanked with the appropriate elution buffer. One 1.5 µl of each sample was measured. The concentration of RNA was showed in ng/ul. For a more detailed description see NanoDrop™ 1000 manual.

3.6 Quantitative reverse transcription polymerase chain reaction

The reverse transcription polymerase chain reaction (RT-PCR) serves to transcribe RNA into cDNA and to amplify the cDNA. In this reaction two enzymatic steps are involved. In the first step, a reverse transcriptase converts the RNA into cDNA. In the second step, in which the cDNA is amplified in a PCR reaction, the enzyme needed is a Taq polymerase. The Taq polymerase is a heat stable enzyme derived from the thermophilic bacterium *Thermus aquaticus (Chien et al., 1976, Saiki et al., 1988)*. There are two different forms of RT-PCR, onestep and two-step. In the one-step RT-PCR everything takes place in the same tube. Because the both enzymatic reactions occur in a single tube, the one-step RT-PCR minimizes experimental variation. However, because RNA is prone to rapid degradation if not handled properly, the on-step method is not suitable for applications in which the same sample is used over a long period of time (Wong and Medrano, 2005). It has also been reported that the one-step RT-PCR is not as sensitive as two-step RT-PCR (Battaglia et al., 1998). The two-step RT-PCR separates the reverse transcription from the PCR

amplification. They take place in different tubes. Because the steps are separated, this allows different real-time PCR reactions from the same cDNA sample. A two-step RT-PCR is highly reproducible and should be preferred when using SYBR green or similar dyes (Vandesompele et al., 2002). Because there are more pipetting steps, the two-step method is more prone to DNA contamination than the one-step method (Wong and Medrano, 2005).

3.6.1 Reverse transcription

The reverse transcriptase allows the transcription of RNA into DNA (Figure 3: Schematic overview of reverse transcription.). The reverse transcriptase was discovered by Howard Temin (Temin and Mizutani, 1970) and David Baltimore (Baltimore, 1970), independently. At that time the existence of a reverse transcriptase was discussed controversially (Crick, 1970). Nowadays reverse transcription is commonly used to transcribe RNA into cDNA for subsequent applications like PCR. This is necessary, because the PCR technique works only with DNA. The reverse transcription can be primed with random primers, oligo(dT), or a gene-specific primer. Random primers are good for the detection of non-coding RNAs, because they can detect all kinds of RNA, they anneal at a random point in the RNA sequence, depending on sequence specificity. Because of their random binding, the cDNA of mRNAs will not be full length. Another disadvantage is that ribosomal RNA will be amplified as well, which may lead in some cases to lower reverse transcription of target mRNA. Oligo(dT) primers have a high specificity for RNAs with a poly(A) tail. The problem can be that the reverse transcription does not reach the 5’ end of long transcripts.

In this study the high-capacity cDNA reverse transcription kit (4368813, Applied Biosystems/Life Technologies, Darmstadt, Germany) was used. Up to 2 µg of total RNA could be used, in a 20 µl reaction, to be converted into single stranded cDNA. The reverse transcriptase used in this kit is the MultiScribe™ MuLV (murine leukemia virus) reverse transcriptase. MuLV reverse transcriptase is a recombinant RNA-dependent DNA polymerase that uses single-stranded RNA as a template in the

presence of a primer to synthesize a cDNA strand. The reverse transcriptase has a weak RNase H activity, which means it can hydrolyze the RNA in RNA:DNA hybrid strands, leaving behind single stranded cDNA. For each sample 10 µl of RNA (up to 2 µg) was used. After the isolation of the RNA, the RNA concentration was measured with the NanoDrop™. If necessary RNA was diluted with RNase-free water so that 10 µl could be used in each reaction without getting over the limit of 2 µg per reaction. A master mix for the required number of reactions was calculated and then prepared (Table 2: Master mix for reverse transcription).

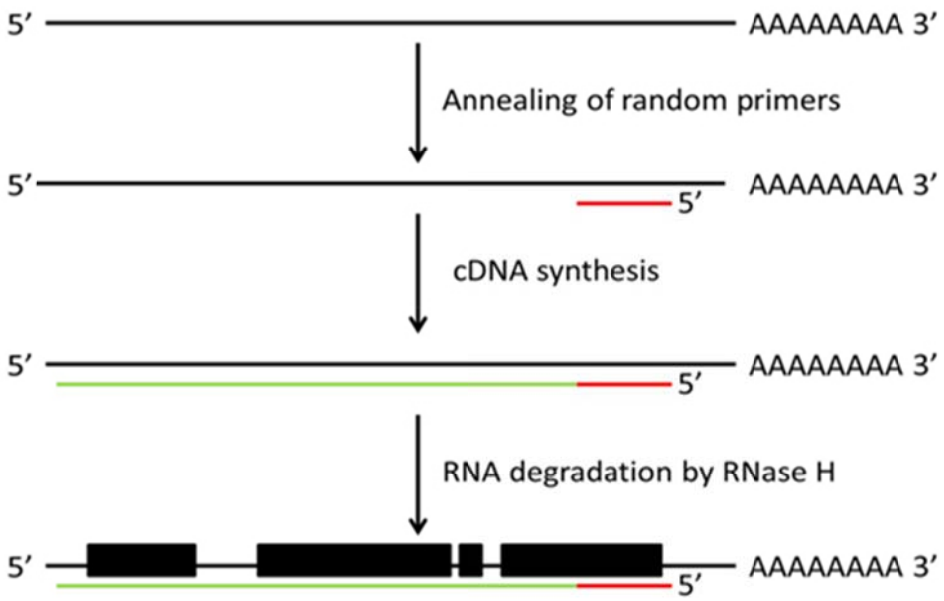

Figure 3: Schematic overview of reverse transcription.

cDNA synthesis occurs in several steps. The random primers (red) anneal, depending on sequence specificity, on a random point of the mRNA (black). After annealing the reverse transcriptase creates a cDNA strand (green). By its RNase H activity the reverse transcriptase itself, degrades the RNA strand of the RNA: DNA hybrid. The result is a single strand cDNA which can be used for qPCR or other downstream applications. Oligo(dT) primers will anneal to the poly(A) tail.

Table 2: Master mix for reverse transcription

Component	Volume/reaction (µl)
10x reverse transcription buffer	2
25x dNTP Mix (100 mM)	0.8
10x random primers	2
MultiScribe™ reverse transcriptase	1
Nuclease-free H_2O	4.2
Total volume per reaction	10

The master mix was placed on ice and the MultiScribe™ reverse transcriptase was added at the last moment, before the mix was added to the RNA. 10 µl of RNA was added into 0.2 ml PCR tubes. The tubes with the RNA were placed on ice. After the addition of the reverse transcriptase to the master mix, the master mix was mixed gently. The 10 µl of the master mix were added to each PCR tube containing the 10 µl RNA, sealed with a cap, mixed and centrifuged. All tubes were then placed in a thermocycler and the program was started to convert the total RNA into single stranded cDNA (Table 3: Thermal cycling conditions for reverse transcription).

Table 3: Thermal cycling conditions for reverse transcription

	Temperature	Time
Step 1	25 °C	10 minutes
Step 2	37 °C	120 minutes
Step 3	85 °C	5 minutes
Step 4	4 °C	∞

3.6.2 Quantitative real-time polymerase chain reaction (qPCR)

The qPCR is an effective method to amplify, simultaneously detect and quantify one or more target DNAs in real-time (Higuchi et al., 1993). The qPCR method works similarly to a normal PCR reaction, with the difference that the amplification of the target(s) can be monitored in real-time with a fluorescence detection system. There are different techniques to generate the fluorescence signal (Mackay et al., 2002). The first option is to use double-stranded DNA binding dyes such as SYBR Green I. SYBR Green I binds to the minor groove of double-stranded DNA, thus causing fluorescence of the dye. Because the amount of double-stranded DNA increases in each PCR cycle, the fluorescence intensity increases proportionally. The increase in fluorescence can be measured and this allows quantification of DNA concentration (Figure 4: Schematic view of SYBR Green I chemistry.). The disadvantage is that SYBR Green I binds not only target double-stranded DNA, but also primer-dimers or other unspecific PCR products, which can interfere with quantification of the target DNA. Another detection opportunity is the probe based method. This technique is more accurate than the SYBR Green I method, because the probe binds only to its specific sequence, thereby avoiding false signals like primer-dimers. By labeling different probes with different dyes, more target genes can be detected simultaneously in a multiplex PCR. The principle is the same as with SYBR Green I; an increase of the specific PCR product leads to an increase in fluorescence signal. The probes are labeled with the fluorescence dye at their 5' end and a quencher at the 3' end. The quencher is necessary to avoid auto-fluorescence of the probe. Only after the degradation of the quencher by the Taq polymerase, the close proximity of the quencher to the dye is broken and fluorescence can be detected (Holland et al., 1991). The negative aspect of the probe based method is that for every target, a new probe has to be designed.

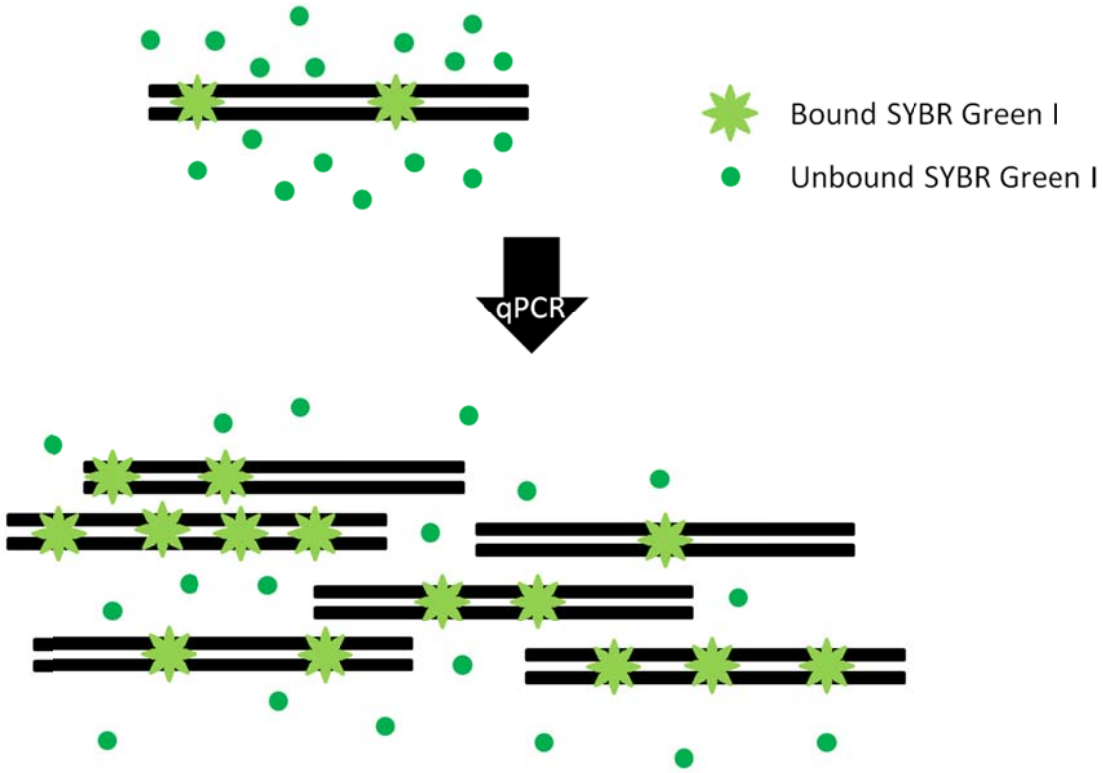

Figure 4: Schematic view of SYBR Green I chemistry.

SYBR Green I binds to double-stranded DNA and thereby increasing its fluorescence. The amplification of new double-stranded DNA during every PCR cycle increases the fluorescence signal, because more SYBR Green I can be bound to the newly synthesized double-stranded DNA products.

Real-time PCR can be quantified in two ways. Either in an absolute quantification, which uses external standards to determine the absolute value of target DNA. But in this study, the relative quantification was used, which calculates the ratio between the target and an internal control. This internal control is normally a housekeeping gene, which should not differ in concentration in various experimental states. Every fluorescence probe has background fluorescence. This is true for SYBR Green I, too. To account for the background fluorescence, a threshold for every DNA-based fluorescence is set over the background. The number of PCR cycles, at which the DNA-based fluorescence is over the background, is called the cycle threshold (C_t). During the exponential amplification phase, the amount of the target DNA doubles every cycle. To calculate the relative expression, the ΔC_t value has to be calculated first. For this, the control C_t is subtracted from the target C_t (C_t target - C_t control).

Then, the ΔC_t value of the treated group is subtracted from the ΔC_t value of the untreated group ($\Delta\Delta C_t$= ΔC_t treated - ΔC_t untreated). Using this method the $\Delta\Delta C_t$ of the untreated group is 0. To calculate the n-fold expression of the target gene the following formula is used:

Fold difference = $2^{\wedge -\Delta\Delta Ct}$

The untreated control is then 1. If for the treated group the $\Delta\Delta C_t$ is -1, this means that the doubled amount of the target gene is expressed under these conditions. If the $\Delta\Delta C_t$ is 1, this means that with this treatment the expression of the target gene is halved. It is important to know that both primer pairs (for the housekeeping gene and for the target gene) should have nearly the same efficiency if using this method.

In our study, the SYBR® Green JumpStart™ Taq ReadyMix™ (S4438, Sigma-Aldrich, Hamburg, Germany) was used. Prior to use the obtained cDNA was diluted with RNase-free water to 10 ng/µl. Because RNA concentration was measured with the NanoDrop™ and 10 µl of RNA was used and the total volume of the reaction was 20 µl, the required quantity of water could be calculated easily. To achieve best reproducibility, a reaction master mix was prepared (Table 4). The sequences of the primers used in this study are shown below (Table 6: Primers used in qPCR for comparison of mRNA expression.). The reference gene was GAPDH because it fulfilled all the characteristics for an acceptable reference gene, i.e. the expression of GAPDH was stable even after treatment. The data were normalized to GAPDH.

The qPCR was performed in a 96-well plate. 4 µl (40 ng) of every sample was placed in a 96-well plate and 16 µl of master mix was added. The plate was sealed with an optical film and spinned down. After this, the plate was placed in a real-time PCR thermal cycler and the following program (Table 5) was used.

If necessary (e.g. in SYBR Green technique), a melt curve was added at the end of the run to analyze the PCR product. Data were analyzed using the software provided with the real-time PCR thermal cycler. Excel was used to calculate the relative expression with the $\Delta\Delta C_t$ method.

Table 4: Master mix for qPCR

Component	Volume/reaction (µL)
2x SYBR® Green Jump Start Taq ReadyMix	10
Forward primer	0.2
Reverse primer	0.2
H_2O	5.6
Total volume per reaction	16

Table 5: Thermal cycling conditions for qPCR

	Temperature	Time	Number of cycles
Initial denaturation	94 °C	2 minutes	1
Denaturation	94 °C	15 seconds	40
Annealing, extension, and read fluorescence	60 °C	1 minute	
Hold	4 °C	∞	

Table 6: Primers used in qPCR for comparison of mRNA expression.

Target	Orientation	Sequence (5'→3')
ICAM	forward	CGGGAGATGAATGGTACCTACAA
	reverse	TGCACATCCCTGGTGATACTC
NF-κB	forward	GCTTACGGTGGGATTGCATT
	reverse	TTATGGTGCCATGGGTGATG
VCAM	forward	GAAGCCGGTCATGGTCAAGT
	reverse	GGTCACCCTTGAACAGTTCTATCTC
Ednra	forward	AACCTGGCAACCATGAACTC
	reverse	ATGAGGCTTTTGGACTGGTG
Ednrb	forward	CCTGATATTTCACGGGCTGT
	reverse	TAAGTGGGCTGTGAGTGCAG

Target	Orientation	Sequence (5'→3')
CAT	forward	ACATGGGTCTGGGACTTCTGG
	reverse	CCATTCGCATTAACCAGCTT
HO-1	forward	TCTATCGTGCTCGCATGAAC
	reverse	GAAGGCGGTCTTAGCCTCTT
ACE	forward	CAGAGGCCAACTGGCATTAT
	reverse	CTGGAAGTTGCTCACGTCAA
ACE2	forward	TGGACGAAATAATGGCAACA
	reverse	TATAACGGCCTCAGCTGCTT
Adra1b	forward	GGTCTTGTCCACGGTCATCT
	reverse	TGACCCCACATTCTTTGTCA
Adra1d	forward	CCGAGGTAGAAGCAGTGTCC
	reverse	GTCAGTCTCTCGGAGGTTGC
Agtr1a	forward	GGAAACAGCTTGGTGGTGAT
	reverse	ATAAGTCAGCCAAGGCGAGA
Agtr1b	forward	GGGGTTAGGGAGGAGGTGTA
	reverse	TGACCTCCCATCTCCTTTTG
Agtr2	forward	CATTGACCTGGCACTTCCTT
	reverse	TGGAGCCAAGTAATGGGAAC
Atp2c1	forward	CTCCTCAACCAGCTGCTACC
	reverse	CTTTGCTTTGCCACATCTGA
GPx1	forward	TCGAACCCGATATAGAAGCCC
	reverse	CACCAAGCCCAGATACCAGG
eNOS	forward	GGAGGTTCACCGCGTGC
	reverse	GACGCTGGTTGCCATAGTGAC
NOX1	forward	ACCCCCTGAGTCTTGGAAGTG
	reverse	GGGTGCATGACAACCTTGGT
NOX2	forward	CTTCTTGGGTCAGCACTGGC
	reverse	GCAGCAAGATCAGCATGCAG
NOX4	forward	CTGTCCTGAACCTCAACTGCAG
	reverse	TGTGATCCGCGAAGGTAAGC

Target	Orientation	Sequence (5'→3')
GCH-1	forward	TGTGTATGGTCATGCGAGGT
	reverse	GAGGAACTCCTCCCGAGTCT
GAPDH	forward	TTCTTGTGCAGTGCCAGCC
	reverse	CGTCCGATACGGCCAAATC
SOD1	forward	GTCGTCTCCTTGCTTTTTGC
	reverse	TCTGCTCGAAGTGAATGACG
SOD2	forward	CCAAAGGAGAGTTGCTGGAG
	reverse	TTGGACTCCCACAGACACAG
SOD3	forward	GACCTGGAGATCTGGATGGA
	reverse	GGACCAAGCCTGTGATCTGT
SIRT1	forward	TTCCTGTGGGATACCTGACTTCA
	reverse	TGGCTTGAGGATCTGGGAGAT

3.7 Western blot

Western blot is a commonly used technique to identify distinct proteins out of a homogenous protein mixture. The protein mixture from tissue or cells is first separated by electrophoresis, transferred to a nitrocellulose membrane or polyvinylidene fluoride membrane and then detected with a specific antibody against the protein of interest. As mentioned, the separation of the proteins is carried out by gel electrophoresis. The proteins can be separated by isoelectric point, molecular weight or charge. A combination of the different separation methods is possible, too. For protein analysis, a polyacrylamide gel electrophoresis (PAGE) is used normally. Polyacrylamide separated the proteins by molecular weight, due to its uniform pore size. Electrophoresis can be done under denaturing conditions, which means the secondary, tertiary and quaternary structures of the proteins are not maintained. The protein structure is determined by hydrophobic interactions, hydrogen bonds and disulfide bonds, just to mention some of these. Under denaturing conditions, normally provoked with sodium dodecyl sulfate (SDS), a strong reducing agent, proteins and other macromolecules lose their natural structure. In their denatured form they can be

separated by molecular weight. In the gel electrophoresis the proteins migrate to the anode, because they are covered with the negatively charged SDS.

Smaller molecules migrate faster than larger molecules through the polyacrylamide gel in an electric field, because they can pass easier through the gel matrix. Higher polyacrylamide content (e.g. 2%) allows a better separation of small molecules, because the pores are thinner, but large molecules migrate slower and their separation might be difficult. By reducing the amount of polyacrylamide (e.g. 0.8%) larger molecules are separated better, because the gel matrix has larger pores, but smaller molecules may migrate out of the gel, because they can pass easily the pores and therefore migrate fast. Depending on the size of the macromolecule of interest, the percentage of the polyacrylamide gel should be chosen. After the separation of the proteins in the electric field, they are transferred to a membrane.

The membrane consists of nitrocellulose or polyvinylidene fluoride. The blotting is necessary to make the proteins accessible to antibodies. Again, an electric current is used to blot the proteins from the gel onto the membrane, thereby keeping the layout of the gel intact, this means the proteins are still separated on the membrane as they were on the gel. Nitrocellulose as well as polyvinylidene fluoride bind proteins non-specific. The membrane is placed close to the anode and the proteins migrate in the electric field to the plus pole. The membrane binds the protein in an unspecific manner by hydrophobic interactions and charge interactions between the protein and the membrane. After the transfer, the membrane is stained with Ponceau S, to verify whether all proteins are transferred evenly to the membrane. Ponceau S is a water soluble dye and can be easily washed away afterwards. Antibodies are used to detect the proteins of interest, but antibodies are proteins, too, i.e. the antibodies can bind to the membrane, because the membrane binds protein in a non-specific manner. To avoid this, blocking solution is used prior to the addition of the antibody. The blocking solution contains bovine serum albumin (BSA) or non-fat dry milk to block the membrane. They interact with the membrane and therefore prevent unspecific binding of the antibodies to the membrane.

Blocking the membrane with the blocking solution diminishes the background and false positive results. The antibody solution is left on the membrane between 60 minutes and overnight. After binding of the primary antibody, the membrane is rinsed with Tris-buffered saline containing Tween 20 as a detergent, to wash unbound primary antibodies away. A second antibody is used to detect the species-specific portion (fragment crystallizable region, Fc region) of the primary antibody. The secondary antibody is linked to biotin or to a reporter enzyme like horseradish peroxidase (HRP). The horseradish peroxidase for example cleaves a chemiluminescent agent, and the product of this reaction produces light, which can be detected on a photographic film. Depending on the amount of protein the produced light is proportional. On the photographic film the target protein appears as a band and can be quantified with computer software.

3.7.1 Protein isolation

In our study, 20 mg aorta of rat was homogenized in liquid nitrogen with mortar. The sample powder was then transferred into a 1.5 ml tube filled with 50 µl of lysis buffer (Table 7: Western blot cell lysis buffer formula). Every component in the lysis buffer has a different effect. Sodium pyrophosphate (NaPPi) is a chelating agent and blocks protease and DNase activity, because it binds metal ions with a 2^+ charge and many proteases and DNases need these metal ions to function. The protease and phosphatase inhibitors instead inhibit the activity of the enzymes. Triton X is a detergent; this means it is an amphipathic molecule. Amphipathic molecules can dissolve fats (like in the cell membrane) by forming micelles, i.e. the hydrophobic part of the amphipathic molecule point toward the fat molecule. Tris-HCl and NaCl (Table 8) are buffering agents and they prevent protein degradation. Sodium fluoride (NaF) is a serine/threonine phosphatase and acidic phosphatase inhibitor. NaF mimics the nucleophilic hydroxyl ion in the active site of the enzyme and thereby inhibiting its activity.

The tube was then incubated on ice for 10 minutes, followed by a centrifugation at 4 °C at 13,000 revolutions per minute (rpm) for 10 minutes. The pellet was discarded and the supernatant was transferred into a new 1.5 ml sample tube.

Table 7: Western blot cell lysis buffer formula

Component	Supplier	Volume (µl) per 1 ml
5x pre-lysis buffer (Table 8)		200
Triton X (10%)	6683, Carl Roth, Karlsruhe, Germany	100
Protease and Phosphatase Inhibitor Cocktail	78442, Thermo Fisher Scientific, Waltham, MA, USA	10
H_2O		690

Table 8: 5x pre-lysis buffer

Component	Supplier	Concentration (mM)*
Tris-HCl, pH 7.5	9090, Carl Roth, Karlsruhe,Germany	20
NaCl	3957, Carl Roth, Karlsruhe,Germany	150
NaPPi		10
NaF		20

*dissolved in H_2O

3.7.2 Bicinchoninic acid assay

The bicinchoninic acid (BCA) assay was used to determine protein concentration (Smith et al., 1985); thereby a color change from green to purple could be seen and measured with colorimetric techniques. Proteins can reduce Cu^{2+} to Cu^{+} in an alkaline medium. Peptides with three or more amino residues form a light blue colored chelate complex with cupric ions. This reaction is temperature depended. The amount of protein in the solution is proportional to the amount of reduced cupric ions. In the next step two molecules of BCA chelate with one cuprous ion, leading to a strong purple-colored complex. The color formation reaction is strongly dependent on amino

acid residues (cysteine or cystine, tyrosine, and tryptophan). The formed purple-colored complex absorbs light at a wavelength of 562 nm. The BCA reaction is 100 times more sensitive than the light blue complex formed by the reduction of Cu^{2+} to Cu^{+} by the proteins. Peptide bonds assist in forming the BCA/copper complex at higher temperatures, which leads to a higher increase assay sensitivity while minimizing the variances caused by unequal amino acid composition (Olson and Markwell, 2007). To determine the protein concentration, the absorption of the sample was compared to the absorption of samples with a known protein concentration.

The protein concentration is measured colorimetric with the Sunrise™ microplate reader (Tecan Group, Männedorf, Switzerland) at a wavelength of 562 nm. The Magellan™ software (Tecan) was used for data analysis. A standard curve of bovine serum albumin Fraktion V (BSA) (K41-001, PAA Laboratories, Cölbe, Germany) was used (Table 9: Standard curve for determination of protein concentration) to determine the protein concentration in each sample. The standard curve is done in replicates, in total 18 wells are needed for the standard curve.

Table 9: Standard curve for determination of protein concentration

Well	1	2	3	4	5	6	7	8	9
BSA (µl, 1 mg/ml)	0	1	2	5	10	20	30	40	50
H_2O (µl)	50	49	48	45	40	30	20	10	0

For each sample, 5 µl sample solution was added to one well, filled up with 45 µl of H_2O. Prior to starting the reaction, a master mix was prepared containing 196 µl BCA and 4 µl $CuSO_4$ per well. 200 µl of BCA/copper solution was added to each well and the plate was placed in an incubator at 37 °C for 30 minutes. Then, the plate was read in the microplate reader.

3.7.3 Gel electrophoresis and immunoblotting

After the protein isolation and determination of the protein concentration, the same amount of protein (normally ≈ 30 µg) from every sample was transferred into a new tube and stored on ice. A 10 % resolving Bis-Tris gel (Table 10: Components for a 10 % resolving gel) was used for electrophoresis. The 10 % resolving is suitable to separate proteins in a range of 20 ~ 150 kilo Dalton (kDa), and all the proteins examined in this study fall into this range. The following proteins were detected: heme oxygenase 1 (HO-1, ≈ 32 kDa), eNOS (≈ 140 kDa), GTP cyclohydrolase 1 (GCH-1, ≈ 35 kDa), sirtuin 1 (SIRT1, ≈ 120 kDa), glutathione peroxidase 1 (GPx1, ≈ 22 kDa), superoxide dismutase 2 (SOD2, ≈ 25 kDa), tubulin (≈ 55 kDa), glyceraldehyde-3-phosphate dehydrogenase (GAPDH, ≈ 35 kDa). The Tris(hydroxymethyl)aminomethane-HCl (Tris-HCl, pH 8.8, 1.5 M) for the gel contains 36.3 g Tris(hydroxymethyl)aminomethane dissolved in H_2O and adjusted to pH 8.8 with HCl. To create the 10 % ammonium persulfate (APS) solution, 10 mg APS are dissolved in 1 ml H_2O.

Table 10: Components for a 10 % resolving gel

Component	Supplier	Volume (µl)
H_2O		7900
Tris-HCl (pH 8.8, 1.5 M)		5000
10% SDS		200
30 % acrylamide	A1672, Applichem, Darmstadt, Germany	6700
10 % APS	A2941, Applichem, Darmstadt, Germany	200
TEMED	A1148, Applichem, Darmstadt, Germany	8

The resolving gel was prepared and filled between a 1.5 mm spacer plate (165-3312, Bio-Rad Laboratories, Hercules, CA, USA) and a short plate (165-3308, Bio-Rad Laboratories, Hercules, CA, USA); the glass plates were fixed in a casting frame (165-3304, Bio-Rad Laboratories, Hercules, CA, USA) on a casting stand (165-3303,

Bio-Rad Laboratories, Hercules, CA, USA) prior to gel casting. The resolving gel was left for polymerization.

Table 11: Components for a stacking gel

Component	Volume (µl)
H_2O	5500
Tris-HCl, pH 6.8, 1 M	1000
10% SDS	80
30 % acrylamide	1300
10 % APS	80
TEMED	8

When the resolving gel has polymerized, a stacking gel (Table 11) was cast on the resolving gel and a comb was placed between the spacers into the still liquid stacking gel. Again the gel was left for polymerization. The proteins of the samples were mixed in a 1:1 ration with 2x Laemmli buffer (Table 12: 2x Laemmli buffer), heated for 10 minutes at 95 °C and spinned down at 4°C. The polymerized gels were transferred into gel-running chamber (Bio-Rad Laboratories, Hercules, CA, USA). The chamber was filled with 1x running buffer, which was obtained from 10x running buffer (Table 13) by dilution 1:10 with water.

Table 12: 2x Laemmli buffer

Component	Concentration
Tris-HCl, pH 6.8	0.125 M
Sodium dodecyl sulfate	2%
Glycerol	20%
2-mercaptoethanol	10%
bromphenol blue	0.03%

Table 13: Components for 10x running buffer

Component	Supplier	Quantity	Final Concentration
Tris base	4855,Carl Roth, Karlsruhe, Germany	30 g	250 mM
Glycine	A1067, Applichem, Darmstadt, Germany	144 g	2 M
SDS	2326, Carl Roth, Karlsruhe, Germany	10 g	1%
H_2O		1000 ml	

The samples were loaded on the gel. A PageRuler™ Prestained Protein Ladder (SM0671, St. Leon-Rot, Germany) was used as a reference to determine protein size. The chamber was placed on ice and the electrophoresis was performed with 80 V until the running front reached the end of the glass plate. Then, the gel was removed from the glass plates and the stacking gel was cut off. In the next step, the proteins were transferred on a nitrocellulose membrane (Whatman™ Protran BA 83, GE Healthcare, Little Chalfont, UK); therefore a transfer sandwich was prepared containing soak sponges and blotting paper. The nitrocellulose membrane, soak sponges and blotting paper are equilibrated in transfer buffer (Table 14) prior to use.

Table 14: 20x transfer buffer

Component	Amount (g) for 500 ml
Bicine	40.8
Bis-Tris	52.32
EDTA, disodium	3.8
When diluting to 1 x, include 20 % (final) methanol (4627, Carl Roth, Karlsruhe, Germany)	

The blotting sandwich was prepared as follows: the basis was built by a plastic grid. On the grid, two soak sponges and three layers of blotting paper were placed. On the blotting paper, the nitrocellulose membrane was placed and the gel was carefully laid on the nitrocellulose membrane. The membrane was covered with three layers of blotting paper and two pieces of soak sponge. To ensure stability, a second grid is put on the top of the whole sandwich. Air bubbles were rolled out with a glass bar. The blotting sandwich was then placed in a blotting chamber with the nitrocellulose membrane closest to the positive electrode (anode, red). The blotting chamber was filled with 1x transfer buffer containing 20% (final) methanol. The transfer is carried out over night at 4 °C at 45 V. On the next day, the membrane was stained in Ponceau S (0.5 g Ponceau S, 1 ml acetic acid filled up with H_2O to 100 ml) for five minutes and then rinsed with water to make the bands visible. In the next step, the gel was cut into pieces, i.e. unnecessary parts are discarded. To prevent unspecific reaction of the primary antibody with the membrane, the membrane was placed in blocking solution (5 % skim milk powder in Tris-buffered saline + Tween 20 (TBST, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl with 0.1% Tween 20) for one hour. The antibodies (Table 15) were diluted in 5 % skim milk powder in TBST in dilutions as stated. The antibody solution was left on the membrane at 4 °C overnight in a covered bin. On the next day, the antibody solution was removed and saved (stored at -20 °C until further use) and the blots were washed several times in TBST for five minutes each. After the washing steps, the secondary antibody solution (5 % skim milk in TBST with anti-rabbit IgG HRP-linked antibody) was added to the blot and incubated on an orbital shaker for one hour at room temperature. Then, the antibody solution was discarded and the blot was again washed for several times with TBST for five minutes.

Table 15: Antibodies used for Western blot analyses.

Target	Supplier	Dilution
Rabbit anti-HO1	2322, Epitomics, Burlingame, CA,USA	1 : 1,000
Mouse anti-eNOS	610297, BD, Heidelberg, Germany	1 : 1,000
Mouse anti-GCH-1	2643, Abnova, Heidelberg, Germany	1 : 1,000
Rabbit anti-SIRT1	2028, Cell Signaling, Danvers, MA, USA	1 : 1,000
Rabbit anti-GPx1	3120, Epitomics, Burlingame, CA,USA	1 : 1,000
Rabbit anti-SOD2	SOD110, Enzo, Farmingdale, NY, USA	1 : 1,000
Rabbit anit-tubulin	T7816, Sigma-Aldrich, Steinheim, Deutschland	1 : 20,000
Rabbit anti-GAPDH	2251, Epitomics, Burlingame, CA,USA	1 : 20,000
Goat anti-rabbit	A9169, Sigma-Aldrich, Steinheim, Deutschland	1 : 5,000
Rabbit anti-mouse	A9044, Sigma-Aldrich, Steinheim, Deutschland	1 : 5,000

The last washing step, before detection of the bands on a photographic film, was performed with TBS. After that, the membrane was removed from the bin, drained and placed on a glass plate. The membrane was then covered with Western Lightning Plus-ECL enhanced chemiluminescence substrate (NEL105001EA, Perkin Elmer, Waltham, MA, USA). The Western Lightning solution contains two components (Enhanced Luminol Reagent and Oxidizing Reagent), which were mixed in a 1:1 ratio and incubated for one minute prior to use. In a dark room, the Amersham Hyperfilm ECL (28906837, GE Healthcare, Little Chalfont, United Kingdom) was placed on the membrane as long as necessary to get clear bands of the target proteins. The hyperfilm was placed in Kodak GBX developer solution (5158621, Kodak, Rochester, NY, USA) until black bands appeared, washed in water und finally placed in Kodak GBX fixer solution (5158639, Kodak, Rochester, NY, USA) for a couple of minutes. After fixation, the film was washed again and dried. Densitometric analysis was performed using the Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA).

3.8 Statistical Analysis

Results are expressed as mean±SEM (standard error of the mean). Student's *t* test was used for comparison of two groups. Analysis of variance (ANOVA) followed by Fisher's protected least significant difference test was used to compare mean values between three or more groups. P values < 0.05 were considered significantly different. For statistical analysis GraphPad Prism (GraphPad Software, La Jolla, CA, USA) was used.

4 Result

4.1 Effect of PETN treatment on F0 rats

In the second experiment of our study, blood pressure, heart rate and body weight of F0 rats were measured before breeding and after weaning. The control group was fed with standard chow ad libitum, and the PETN group was fed with PETN chow ad libitum. Approximately 50 mg/kg/day PETN was consumed by pregnant dam. The blood pressure and heart rate were measured by non-invasive tail cuff method. Meanwhile, body weight was monitored. Before breeding, there was no difference between control F0 rats and PETN F0 rats (Figures 5 & 6), considering blood pressure, heart rate and body weight. Systolic, diastolic and mean blood pressure were higher after weaning than before breeding (Table 16), without any difference between control group and PETN group in F0 female rats. Heart rate of both groups did not change during the experiment, and no difference between two groups was found. Due to growth, F0 rats got heavier. The weight gain of PETN females was slower than control female rats (Table 16). It is uncertain whether this phenomenon was caused by potential side effect of PETN; such an effect was not observed in male rats.

		Female		Male	
		Control	PETN	Control	PETN
Before breeding	SBP (mmHg)	168.9 ± 4.1	168.8 ± 5.6	195.6 ± 3.1	196.3 ± 4.7
	DBP (mmHg)	127.2 ± 5.4	123.6 ± 5.0	148.9 ± 3.6	149.4 ± 5.1
	MBP (mmHg)	140.7 ± 4.9	138.4 ± 5.2	164.2 ± 3.2	164.7 ± 5.0
	HR (bpm)	406.5 ± 5.4	400.9 ± 11.5	402.6 ± 10.0	380.6 ± 15.8
	BW (g)	174.8 ± 4.7	179.3 ± 5.5	279.8 ± 3.3	290.5 ± 4.8
After weaning	SBP (mmHg)	170.2 ± 10.1	169.8 ± 4.0	209.2 ± 5.2	201.9 ± 4.1
	DBP (mmHg)	125.9 ± 8.4	127.2 ± 4.5	163.1 ± 5.8	156.6 ± 5.5
	MBP (mmHg)	140.4 ± 8.8	141.0 ± 4.2	178.2 ± 5.4	171.4 ± 5.0
	HR (bpm)	332.2 ± 11.0	348.0 ± 13.6	391.0 ± 16.9	387.2 ± 9.0
	BW (g)	266.5 ± 5.6	238.0 ± 8.9*	358.4 ± 15.3	348.0 ± 14.0

Table 16: Blood pressure, heart rate and body weight of F0 rats before breeding and after weaning.

Spontaneously hypertensive rats (SHR) were orally treated with PETN (about 50 mg/kg/day) during pregnancy and lactation periods. Blood pressure and heart rate were measured before breeding and after weaning non-invasively with the CODA monitor. SBP, systolic blood pressure; DBP, diastolic blood pressure; MBP, mean blood pressure; HR, heart rate; BW, body weight. Data were expressed as mean±SEM. Control group, n=5. PETN group, n=5. * P value <0.05, between control and PETN group.

4.2 Effect of PETN maternal treatment on blood pressure in F1 rats

In the first experiment, the control F0 rats were fed with standard chow ad libitum, and the PETN rats were fed with PETN chow ad libitum, resulting in a dose of approximately 50 mg/kg/day PETN for the pregnant dam. All of the F0 rats were able to complete the gestation period. Total 26 F1 offspring were delivered in control group (15 male rats, 11 female rats), and 21 F1 offspring were delivered in PETN group (13 male rats, 8 female rats). F0 parents in PETN group continued to receive PETN chow diet in lactation period. F1 offspring were allowed to stay with their parents until weaning. After weaning, F1 rats were divided by gender and group, and received standard chow diet ad libitum. Body weight, blood pressure and heart rate were measured at the age of 6 and 8 months, respectively. The average systolic blood pressure of F1 female rats in PETN group was about 13 mmHg and 10 mmHg lower than that of control group at 6 and 8 months, respectively (Figure 5). PETN treatment also reduced the diastolic and mean blood pressure of F1 female rats (Figure 5). No difference was found in heart rate or body weight between female F1 animals. In male F1 rats, no difference was found between PETN and control groups; neither in blood pressure nor in body weight (Figure 6).

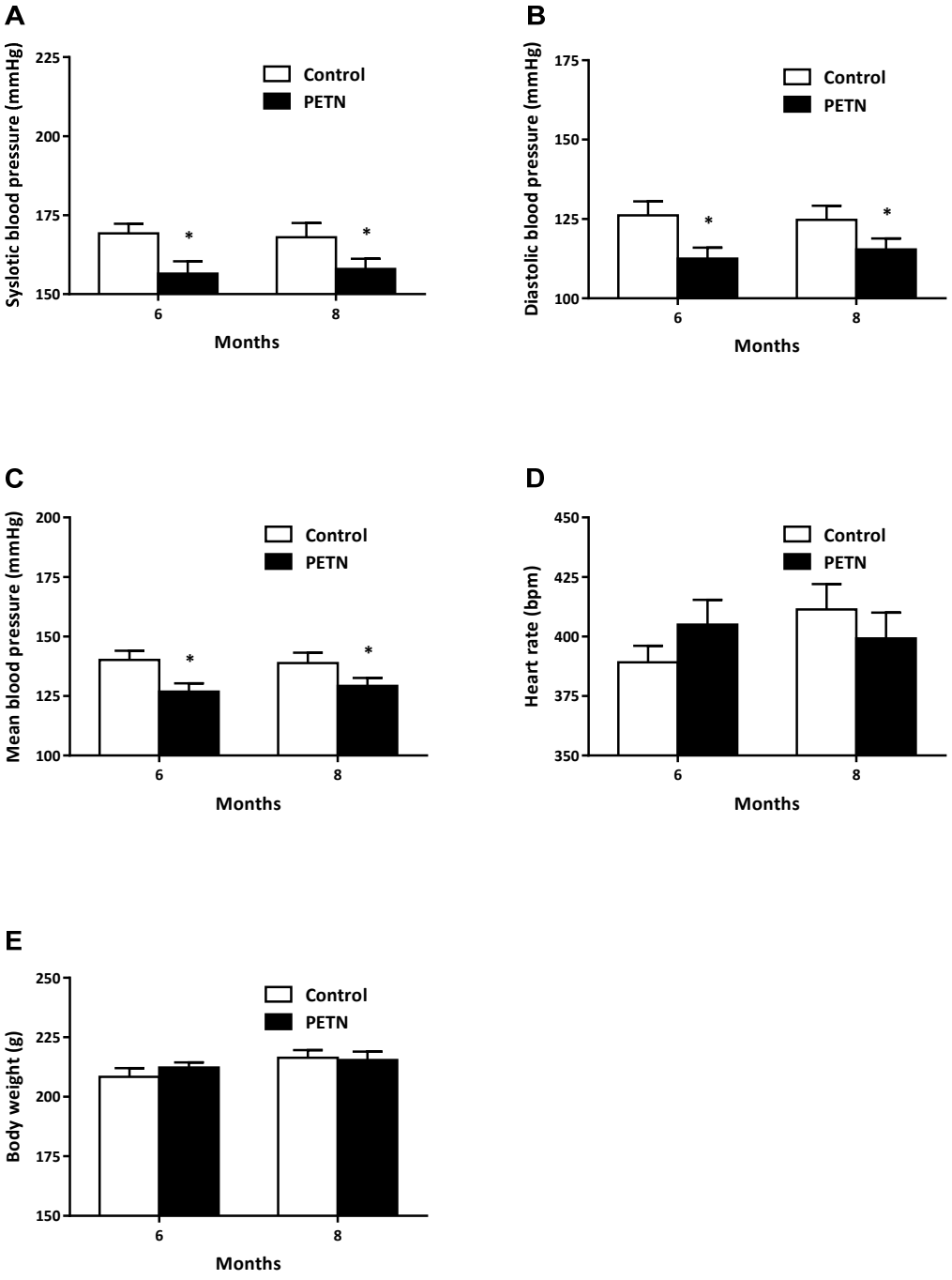

Figure 5: Effect of maternal PETN treatment on blood pressure, heart rate and body weight of F1 female rats (first experiment).

F0 animals were treated with PETN during pregnancy and lactation. Blood pressure, heart rate and body weight were measured in F1 female rats at the age of 6 and 8 months, respectively. Columns represent mean±SEM. n=11 (Control) or 8 (PETN). * P <0.05, compared to Control.

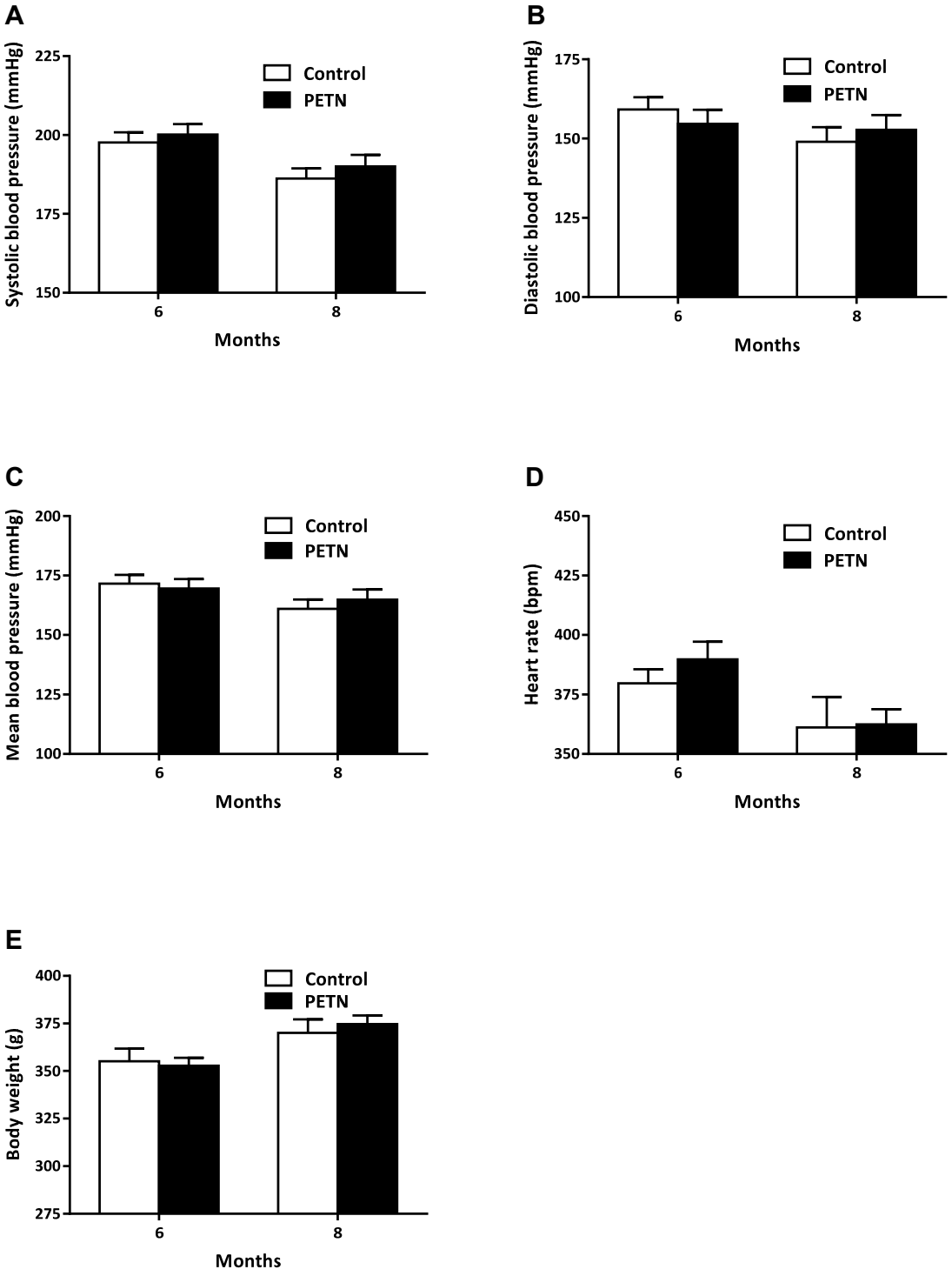

Figure 6: Effect of maternal PETN treatment on blood pressure, heart rate and body weight of F1 male rats (first experiment).

F0 animals were treated with PETN during pregnancy and lactation. Blood pressure, heart rate and body weight were measured in F1 male rats at the age of 6 and 8 months, respectively. Columns represent mean±SEM. n=15 (Control) or 13 (PETN).

In the second experiment, total 41 F1 offspring were born in control group (16 male rats, 25 female rats), and 45 F1 offspring were born in PETN group (19 male rats, 26 female rats). F0 rats in PETN group continued to receive PETN chow diet in the lactation period. F1 offspring were allowed to stay with their parents until weaning. After weaning, F1 rats were divided by gender and group, and received standard chow diet ad libitum. The blood pressure of F1 female rats showed no difference between groups at 3 months. At the age of 4 and 5 months, the blood pressure in F1 females was significantly lower in the PETN group compared to the Control (Figure 7). No difference in heart rate or body weight was found in F1 female rats (Figure 7).

In male F1 rats, the blood pressure in the PETN group tended to be lower; but the difference was not statistically significant (Figure 8). A lower body weight was found in male F1 rats of the PETN group at the age of 4 months (Figure 8).

The second experiment largely confirmed the findings from the first experiment. The effect was stronger in F1 female rats than in the males. For this reason, F1 female rats were used for the following experiments.

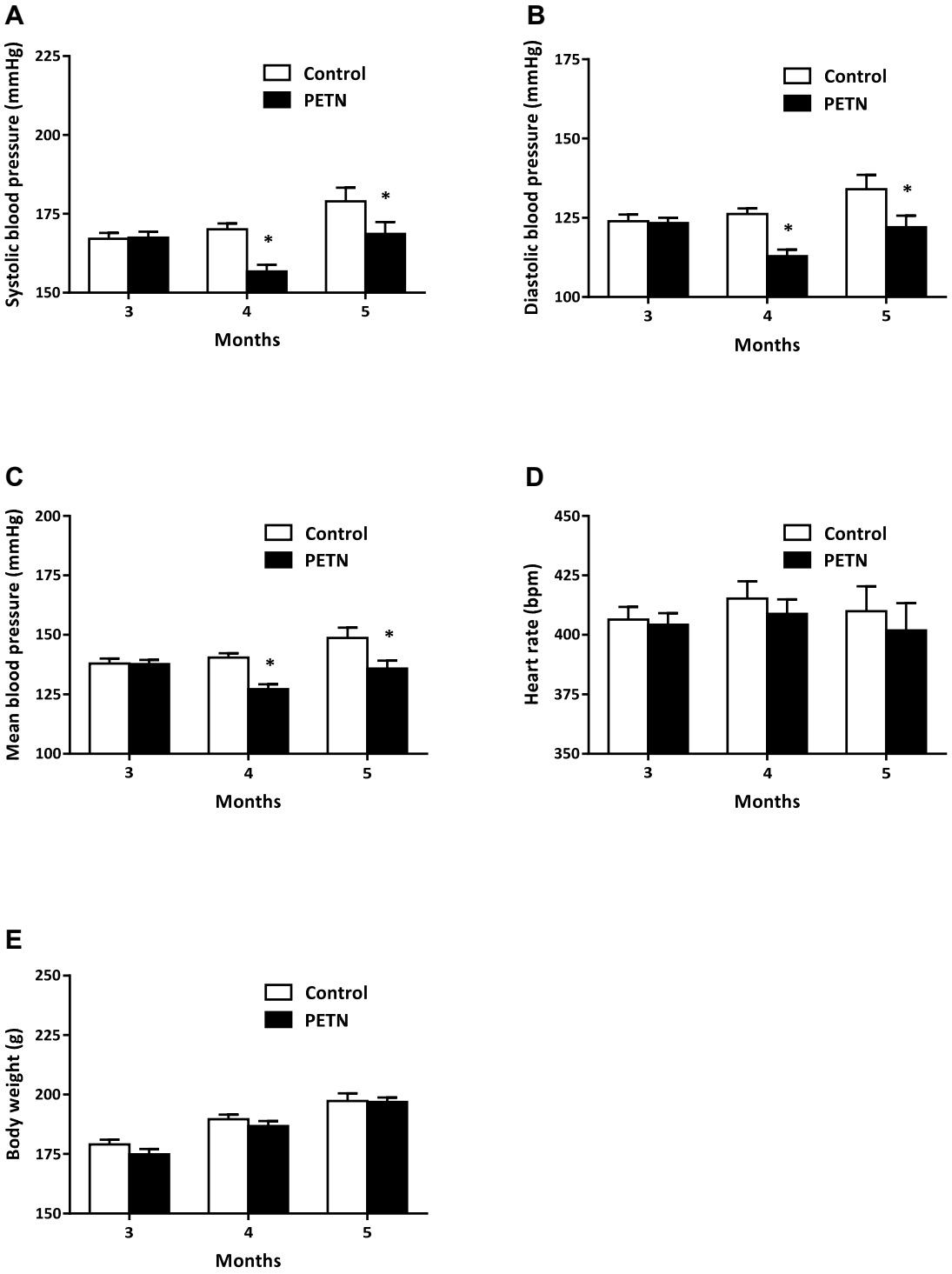

Figure 7: Effect of maternal PETN treatment on blood pressure, heart rate and body weight of F1 female rats (second experiment).

F0 animals were treated with PETN during pregnancy and lactation. Blood pressure, heart rate and body weight were measured in F1 female rats at the age of 6 and 8 months, respectively. Columns represent mean±SEM. n=25 (Control) or 26 (PETN). * P <0.05, compared to Control.

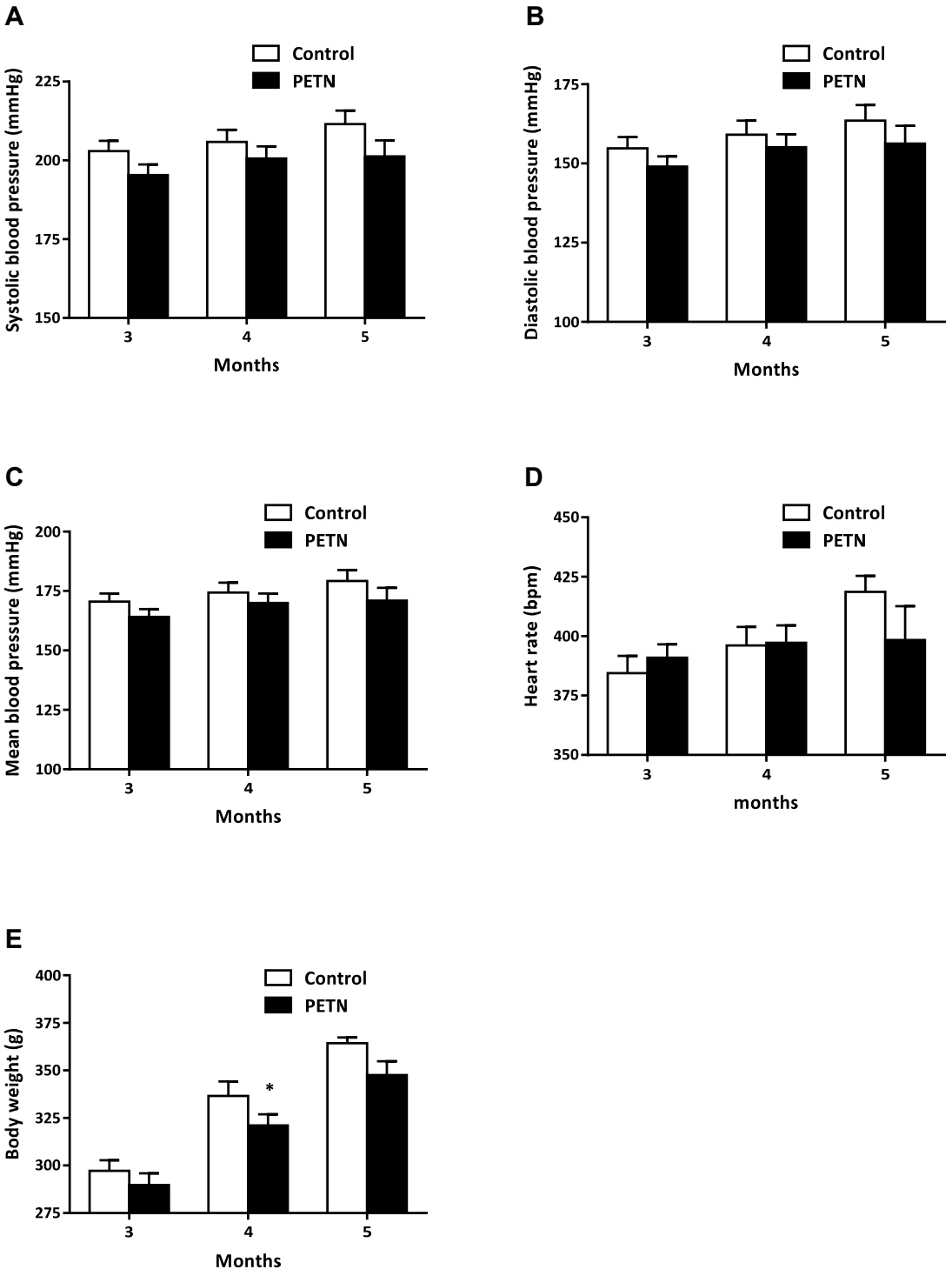

Figure 8: Effect of maternal PETN treatment on blood pressure, heart rate and body weight of F1 male rats (second experiment).

F0 animals were treated with PETN during pregnancy and lactation. Blood pressure, heart rate and body weight were measured in F1 male rats at the age of 6 and 8 months, respectively. Columns represent mean±SEM. n=16 (Control) or 19 (PETN). * P <0.05, compared to Control.

4.3 Effect of maternal PETN treatment on eNOS and GCH-1 in F1 rats

In the first experiment, the F1 rats were sacrificed at 8 months. The aorta samples of F1 female rats were used for quantitative real-time RT-PCR (qRT-PCR). The mRNA expression of eNOS in PETN group was higher than that in control group (Figure 9). The upregulation of eNOS was also confirmed Western blot, which showed higher eNOS protein levels in PETN group. The GTP cyclohydrolase 1 (GCH-1) gene encodes GCH-1 protein, which is the first and rate-limiting enzyme in tetrahydrobiopterin (BH_4) biosynthesis, catalyzing the conversion of GTP into 7,8-dihydroneopterin triphosphate. BH_4 is an essential cofactor required by aromatic amino acid hydroxylases as well as NO synthases. The mRNA expression and the protein expression of GCH-1 were upregulated in PETN group (Figure 9).

Based on the upregulation of eNOS and GCH-1, we expected enhanced NO production and improved endothelial function in the PETN group. This was functionally tested in the Myograph experiment. For this purpose, F1 female rats were sacrificed at 4 months in second experiment, and the aorta was harvested and prepared for the organ bath. After aorta reached 80% of max constriction by adding norepinephrine, acetylcholine was added to induce relaxation. The aorta of PETN group relaxed much faster and better than control group. The maximum relaxations were 95.31±1.85% in PETN group and 81.23±6.64% in control group, respectively ($P<0.05$) (Figure 9). This indicated that the endothelial function of PETN group was better than that of control group. In contrast, the relaxation to SNP, a NO donor, was not different between control and PETN groups (Figure 9).

To verify the role of NO in acetylcholine-induced vasodilation, the aorta was preincubated with NO synthase inhibitor N^{ω}-Nitro-L-arginine methyl ester (L-NAME, 500 μmol/L). The constriction with norepinephrine was enhanced in both control and PETN groups (Figure 10 A versus Figure 11C). Ach-induced vascular relaxation was largely abolished in control and PETN groups (Figure 10).

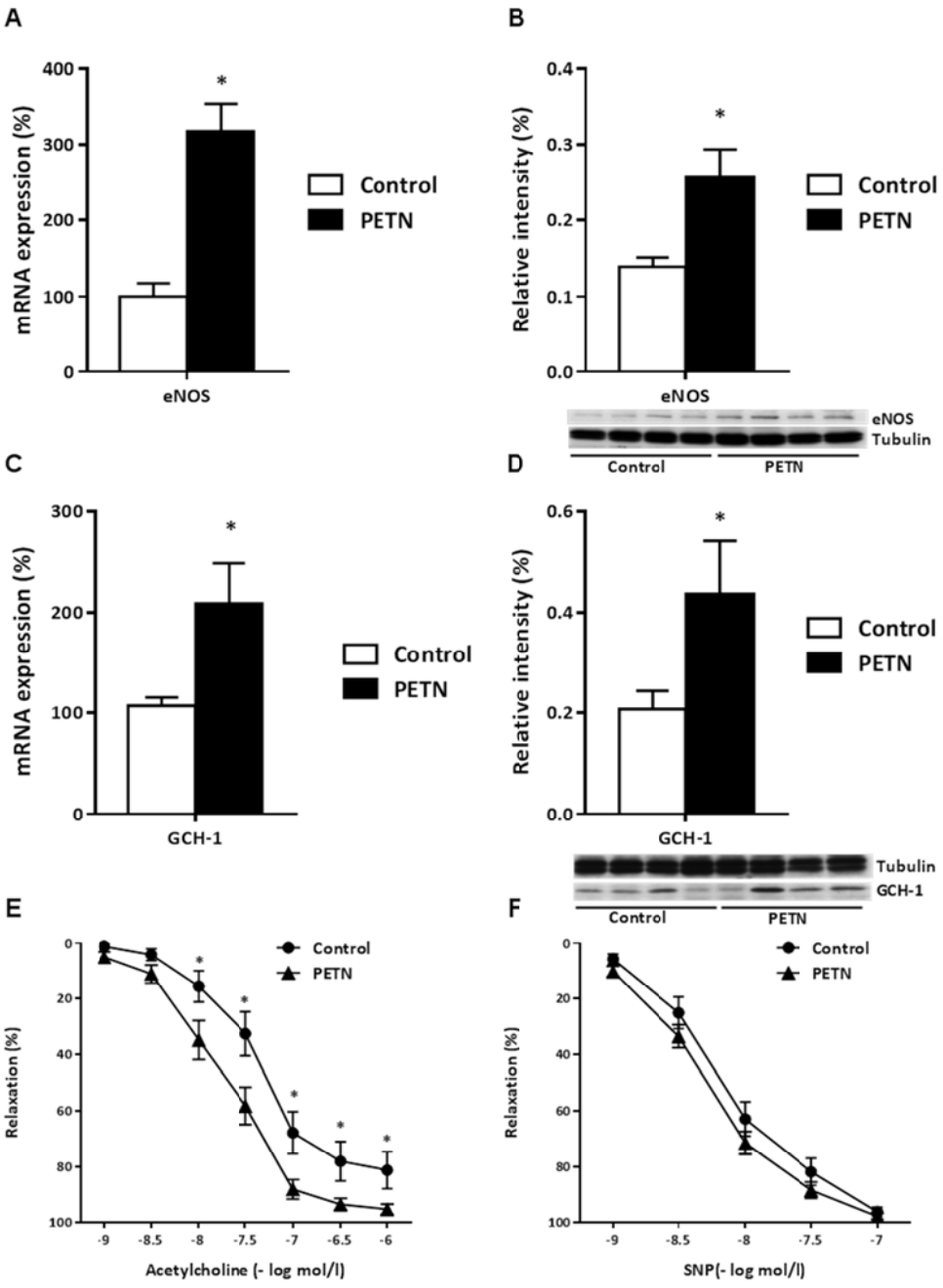

Figure 9: Effect of maternal PETN treatment on eNOS and GCH-1 in F1 female rats.

F1 female rats were sacrificed at the age of 8 months in first experiment, and the aorta was harvested for analyses of mRNA expression with quantitative real-time RT-PCR and protein expression with Western blot, respectively. The relative expression of eNOS mRNA (A), eNOS protein (B), GCH-1 mRNA (C) and GCH-1 protein (D) are shown. n=11 (Control) or 8 (PETN). In the second experiment, F1 female rats were sacrificed at 4 months, and the aorta was isolated for the vascular reactivity study (E and F). The aortic rings were precontracted with norepinephrine (NE; $3*10^{-8}$ mol/L), and then relaxed with acetylcholine (Ach; E) or sodium nitroprusside (SNP; F). n= 11 (Control) or 12 (PETN). Data are presented as mean±SEM. * P <0.05, between Control and PETN.

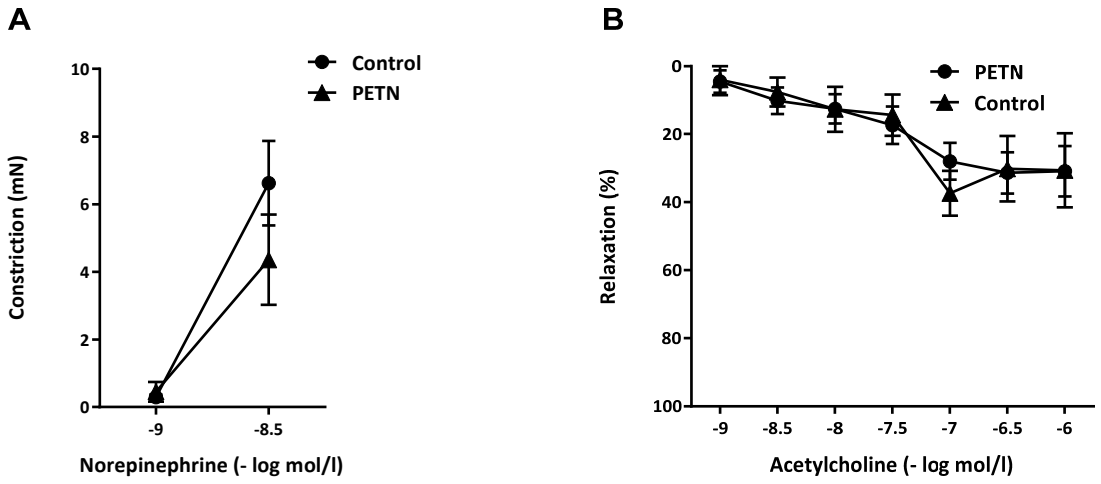

Figure 10: Vasomotor response in the presence of the NO synthase inhibitor L-NAME.

The F1 female rats were sacrificed at 4 months, and the aorta was harvested and prepared for the vascular reactivity study. The aorta was incubated with 500 µmol/L N^{ω}-Nitro-L-arginine methyl ester (L-NAME) for 30 minutes to inhibit eNOS. (A), contractile response to norepinephrine. (B), relaxation response to acetylcholine in aorta precontracted with norepinephrine ($3*10^{-9}$ mol/L). n=6 (Control) or 8 (PETN). Data represent mean±SEM.

4.4 Effect of maternal PETN treatment on α1 adrenergic receptors in F1 rats

The adrenergic receptors are a class of G protein-coupled receptors that are targets of catecholamines, especially norepinephrine and epinephrine. There are two main groups of adrenergic receptors, α and β. Norepinephrine stimulates α1 receptor, which results in increased intracellular Ca^{2+} and then smooth muscle contraction. α1b and α1d are subtypes of α1 adrenergic receptors encoded by Adra1b and Adra1d genes, respectively. The expression of α1b and α1d was analyzed in the aorta of 8-month-old F1 female rats with qRT-PCR. The results showed a downregulation of Adra1b and an upregulation of Adra1d mRNA expression in the aorta of PETN group (Figure 11).

To test the net effect of PETN on catecholamine reactivity, myograph experiments was performed with the aorta of 4-month-old F1 female rats. The vascular reactivity to norepinephrine was not different between the PETN group and the control group (Figure 11). This result indicated that the α1 receptor-mediated vasoconstriction was not changed by maternal treatment with PETN.

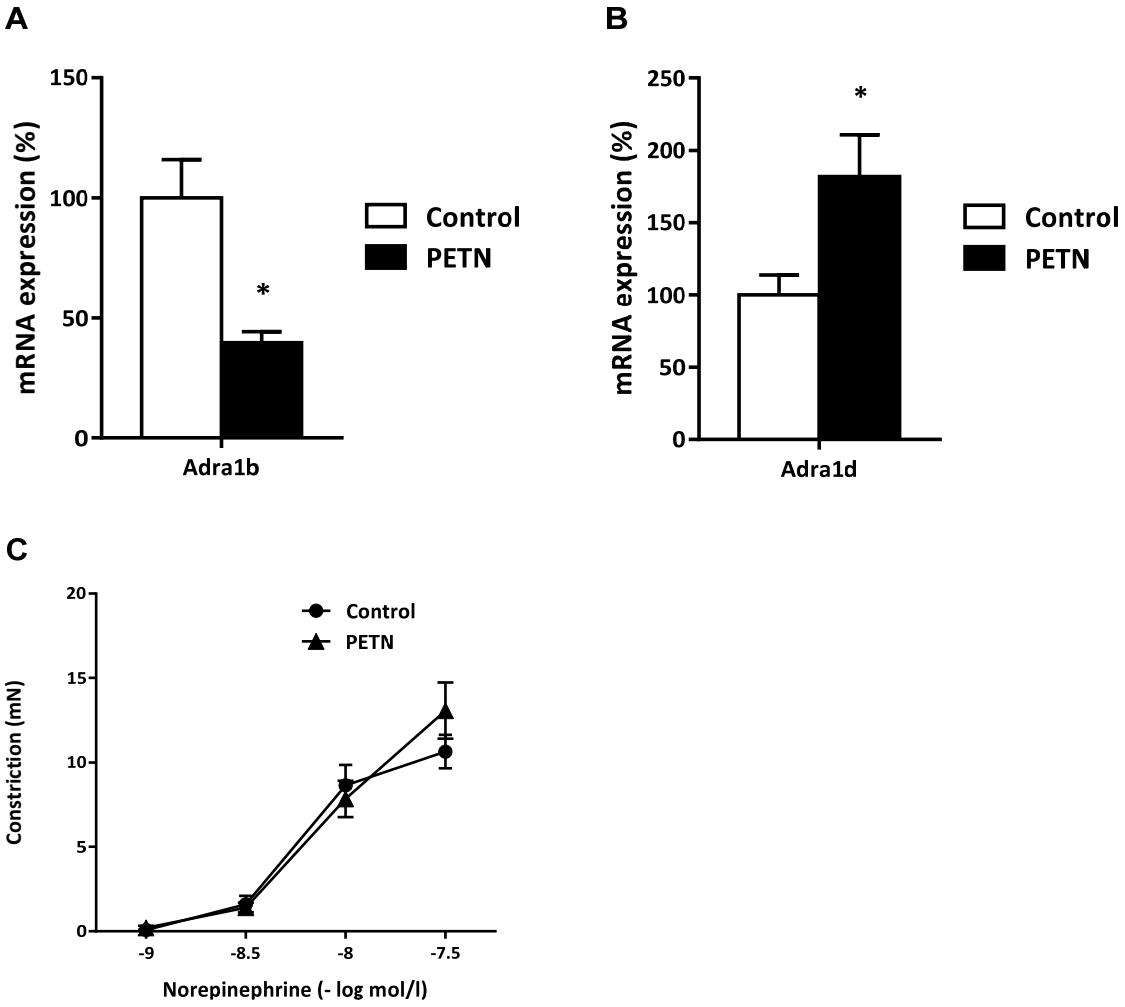

Figure 11: Effect of maternal PETN treatment on α1 adrenergic receptors in female F1 rats

The aorta of 8-month-old female F1 rats was harvested for qRT-PCR experiments (n=11 in Control group; n=8 in PETN group). Aortic rings of 4-month-old female F1 rats were prepared for vascular reactivity study (n=11 in Control group; n=12 in PETN group). (A), mRNA expression of Adra1b; (B), mRNA expression of Adra1d; (C), vascular reactivity to norepinephrine. Data represent mean±SEM. * P <0.05, compared with Control.

4.5 Effect of maternal PETN treatment on RAS components in female F1 rats

RAS is a physiological system that regulates blood pressure and water balance. Renin is an enzyme secreted into blood from kidney; it acts on angiotensinogen to produce angiotensin I, which in turn was converted into angiotensin II (Ang II) by angiotensin-converting enzyme (ACE). Angiotensin II acts as a circulating hormone via angiotensin receptors, angiotensin II receptor type 1 (Agtr1) and angiotensin II receptor type 2 (Agtr2). Agtr1a and Agtr1b are two subtypes of Agtr1. Whereas angiotensin-converting enzyme 2 (ACE2) inactivates angiotensin II and is a negative regulator of the system.

We focused on mRNA expression of ACE, ACE2, Agtr1a and Agtr2, which encoded the corresponding proteins. mRNA expression was analyzed in aorta samples from 8-month-old female F1 rats with qRT-PCR. The results showed no significant difference in mRNA expression of the analyzed genes between PETN and Control (Figure 12).

In vascular reactivity study, no significant difference in contractile response to exogenous angiotensin II was found between PETN and control groups (Figure 12).

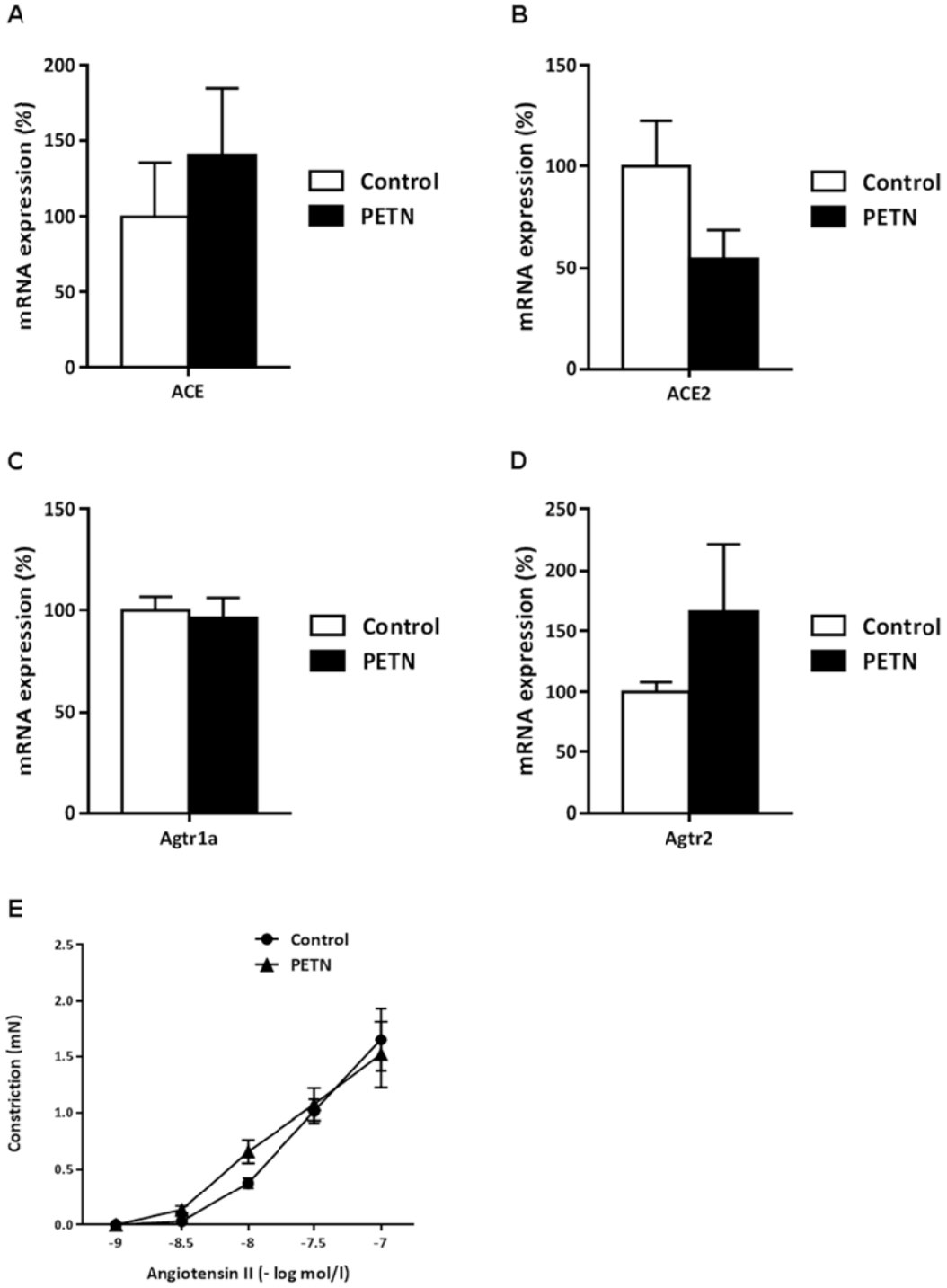

Figure 12: Effect of maternal PETN treatment on RAS components in F1 female rats

The aorta of 8-month-old female F1 rats was harvested for qRT-PCR experiments (A-D; n=11 in Control group; n=8 in PETN group). Aortic rings of 4-month-old female F1 rats were prepared for vascular reactivity study (E; n=11 in Control group; n=12 in PETN group). Data represent mean±SEM. * P <0.05, compared with Control.

4.6 Effect of maternal PETN treatment on endothelin receptor in F1 rats

Endothelins are potent vasoconstrictors and are produced by vascular endothelial cells. There are three isoforms (ET-1, ET-2, ET-3). The vascular effects of ET-1 are mediated by at least two receptor subtypes, endothelin receptor type A (ET_AR) and type B (ET_BR). ET_AR mediates vascular smooth muscle (VSM) contraction. ET_BR in the endothelium mediates the release of relaxing factors such as nitric oxide, prostacyclin and endothelium-derived hyperpolarizing factor, and could also play a role in ET-1 clearance (Mazzuca and Khalil, 2012). ET_AR is encoded by Ednra, while ET_BR is encoded by Ednrb.

In this project, we analyzed the mRNA expression of the endothelin receptors using aorta samples of 8-month-old female F1 rats. The mRNA expression of Ednra was upregulated in PETN group (Figure 13). No significant change was found for Ednrb (Figure 13).

In vascular reactivity study done with aorta from 4-month-old female F1 rats, the contractile response to endothelin 1 tended to be higher in the PETN group (Figure 13). This difference, however, was not statistically significant.

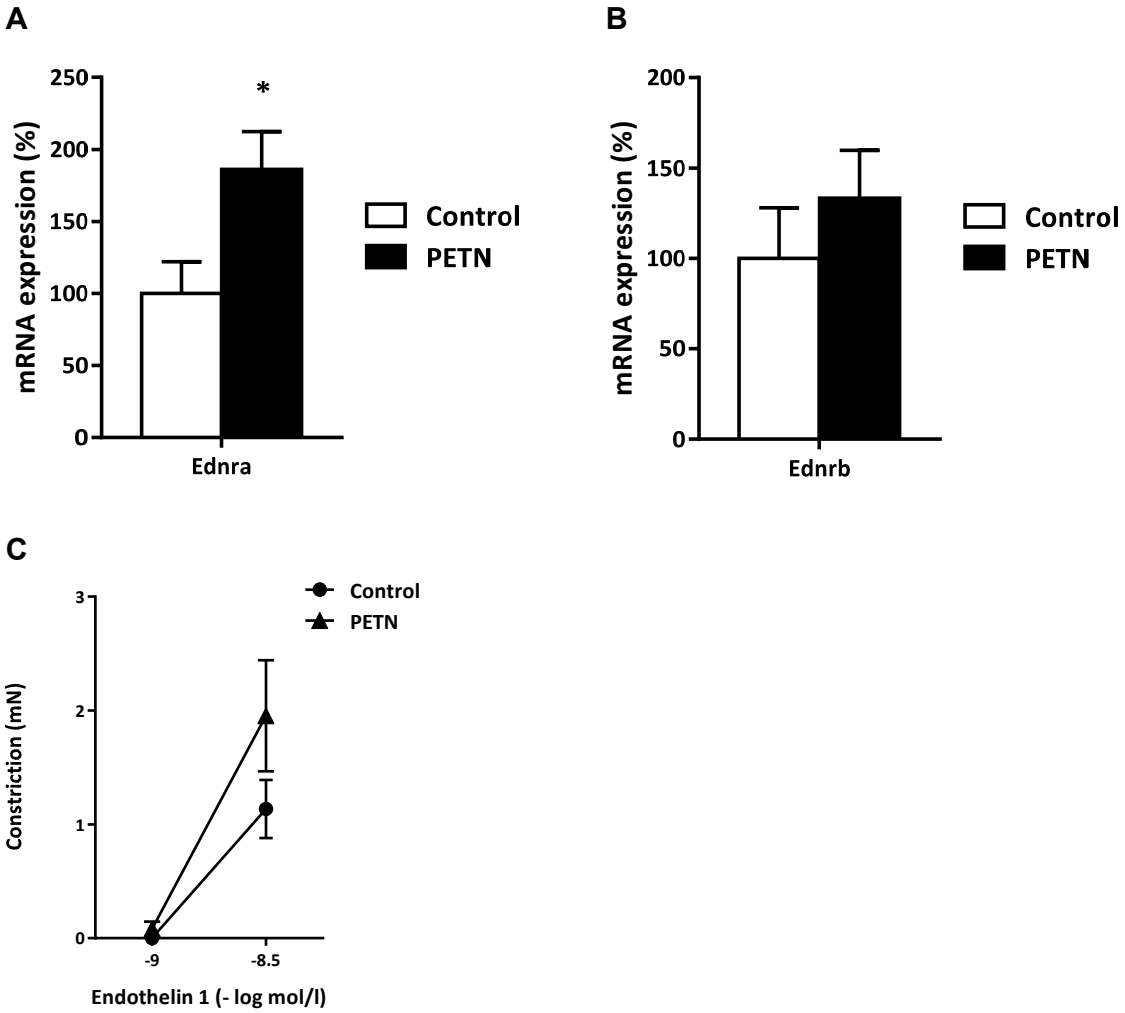

Figure 13: Effect of maternal PETN treatment on endothelin receptor in female F1 rats.

The aorta of 8-month-old female F1 rats was harvested for qRT-PCR experiments (A-B; n=11 in Control group; n=8 in PETN group). Aortic rings of 4-month-old female F1 rats were prepared for vascular reactivity study (C; n=11 in Control group; n=12 in PETN group). Data represent mean±SEM. * P <0.05, compared with Control.

4.7 Effect of maternal PETN treatment on anti-oxidant enzymes in female F1 rats

Heme oxygenase 1 (HO-1) is an isozyme of Heme oxygenase, which is an essential enzyme in heme catabolism, cleaves heme to form biliverdin, subsequently converted to bilirubin by biliverdin reductase, and carbon moNOXide. HO-1 exerts its beneficial effects on vascular function in response to oxidative stress. HO-1 is encoded by HO-1 gene. Glutathione peroxidase 1 (GPx1) is one of the isoenzymes of glutathione peroxidase, which reduces fatty acid hydroperoxides and H_2O_2 at the expense of glutathione, thereby protecting cells against oxidative damage. Catalase (CAT) is a tetrameric haemin-enzyme, it reacts with H_2O_2 to form water and molecular oxygen. Catalase protein is encoded by the CAT gene. Superoxide dismutase (SOD) destroys the free radical superoxide by converting it to H_2O_2 that can in turn be destroyed by CAT or GPx reactions. Three forms of SOD are present in mammals, they are Cu, Zn-SOD (SOD-1), manganese-SOD (SOD2, Mn-SOD), and extracellular-SOD (SOD3, EC-SOD) (Mates and Sanchez-Jimenez, 1999). SOD1 is located in the cytoplasm, SOD2 in the mitochondria, and SOD3 is extracellular.

We analyzed the expression of these anti-oxidant enzymes in the aorta of 8-month-old female F1 rats. The expression of HO-1, GPx1 and SOD2 was upregulated in PETN group at both mRNA and protein levels (Figures 14 & 15). No significant changes were found for CAT, SOD1 or SOD2.

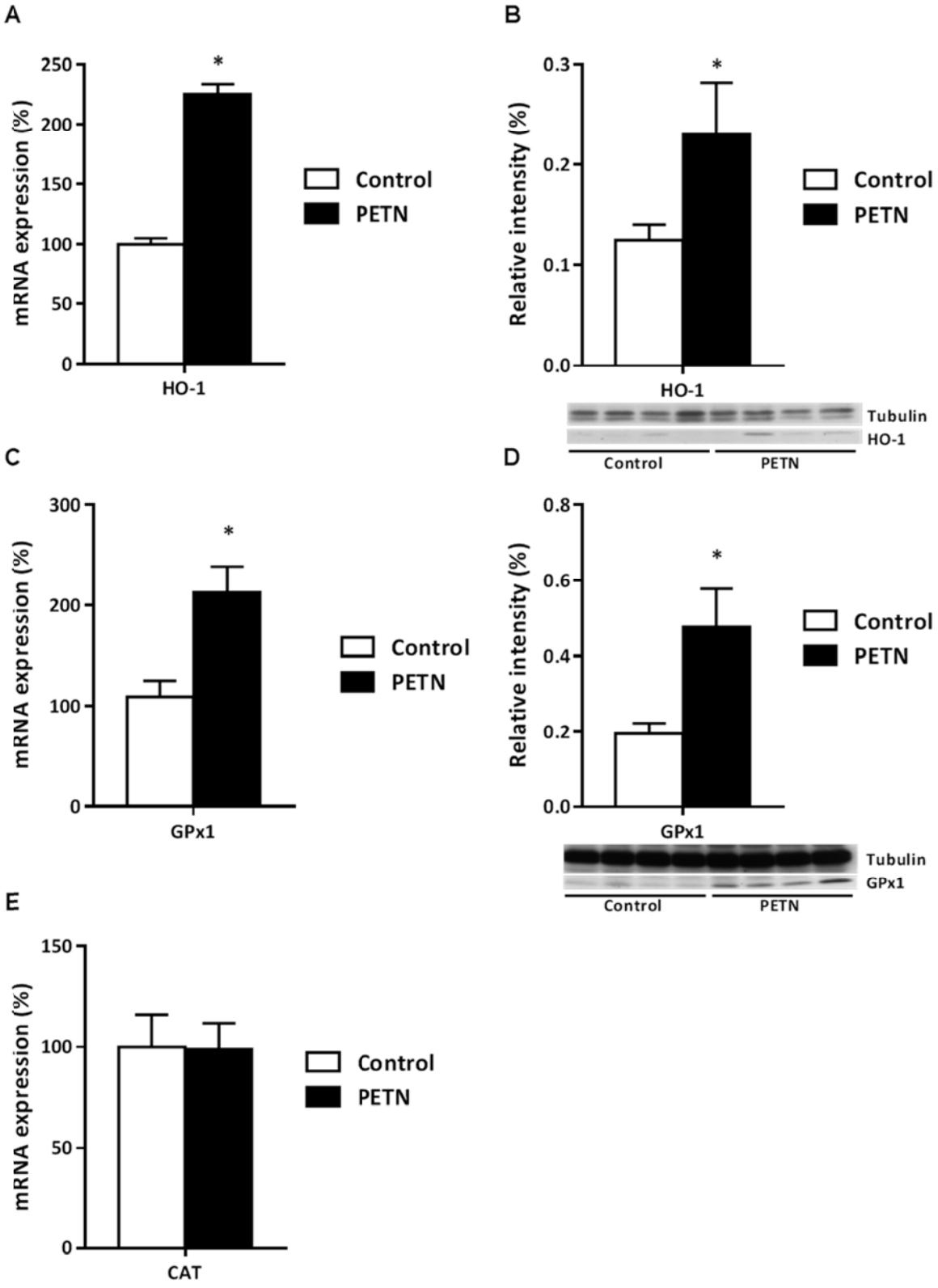

Figure 14: Effect of maternal PETN treatment on HO-1, GPx and CAT in female F1 rats

The aorta of 8-month-old female F1 rats was harvested for qRT-PCR experiments and Western blot analyses (A-E; n=11 in Control group; n=8 in PETN group). Data represent mean±SEM. * P <0.05, compared with Control.

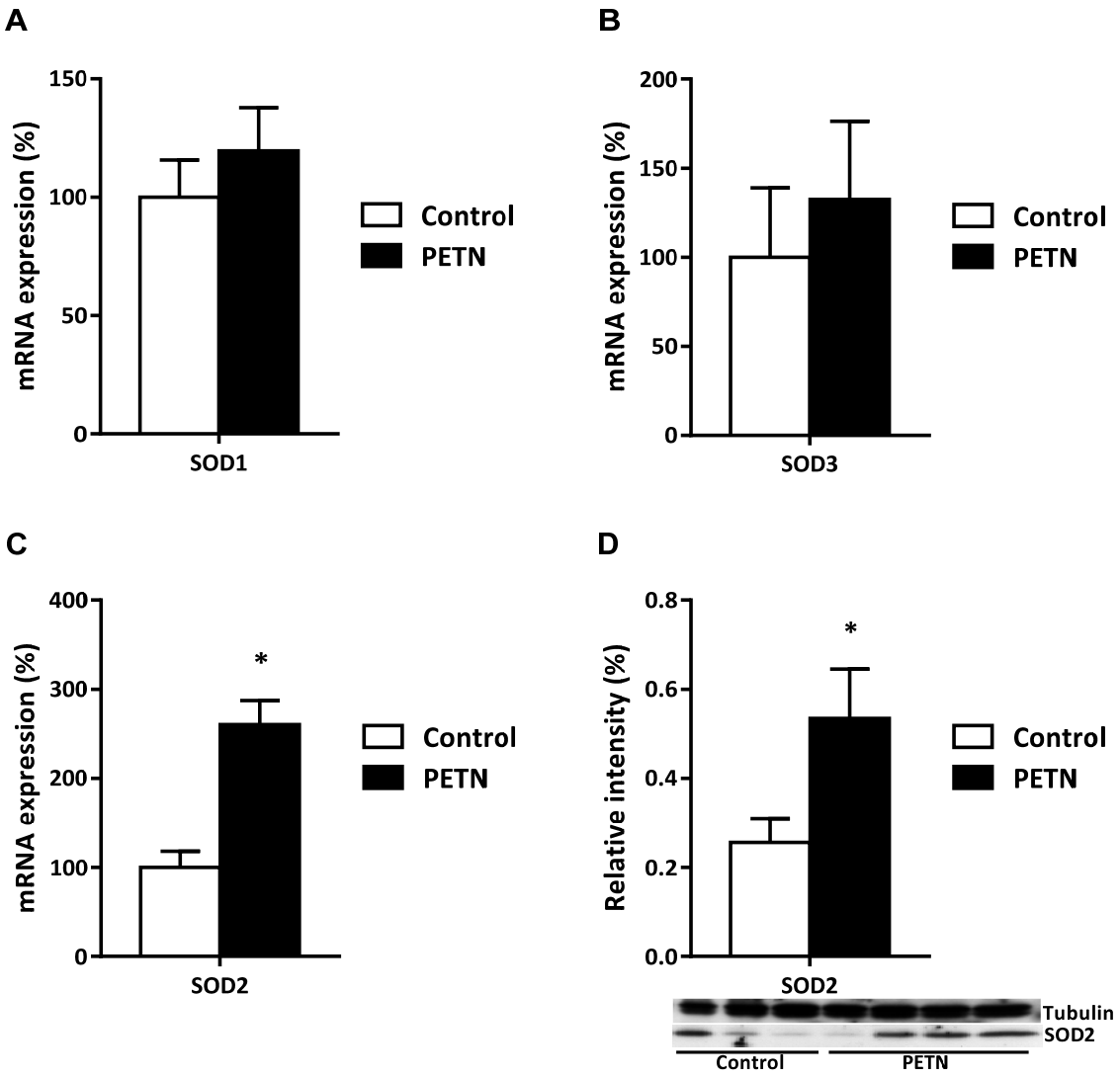

Figure 15: Effect of maternal PETN treatment on SOD isoforms in female F1 rats.

The aorta of 8-month-old female F1 rats was harvested for qRT-PCR experiments and Western blot analyses (n=11 in Control group; n=8 in PETN group). Data represent mean±SEM. * P <0.05, compared with Control.

4.8 Effect of maternal PETN treatment on NADPH oxidases in female F1 rats

NADPH oxidases (NOX) are the major sources of reactive oxygen species (ROS) in vascular physiology and pathophysiology. NOX-1, -2 and -4 are the catalytic homologues of NADPH oxidases family, and are encoded by NOX1, NOX2 and NOX4 genes.

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 were not changed by PETN treatment (Figure 16).

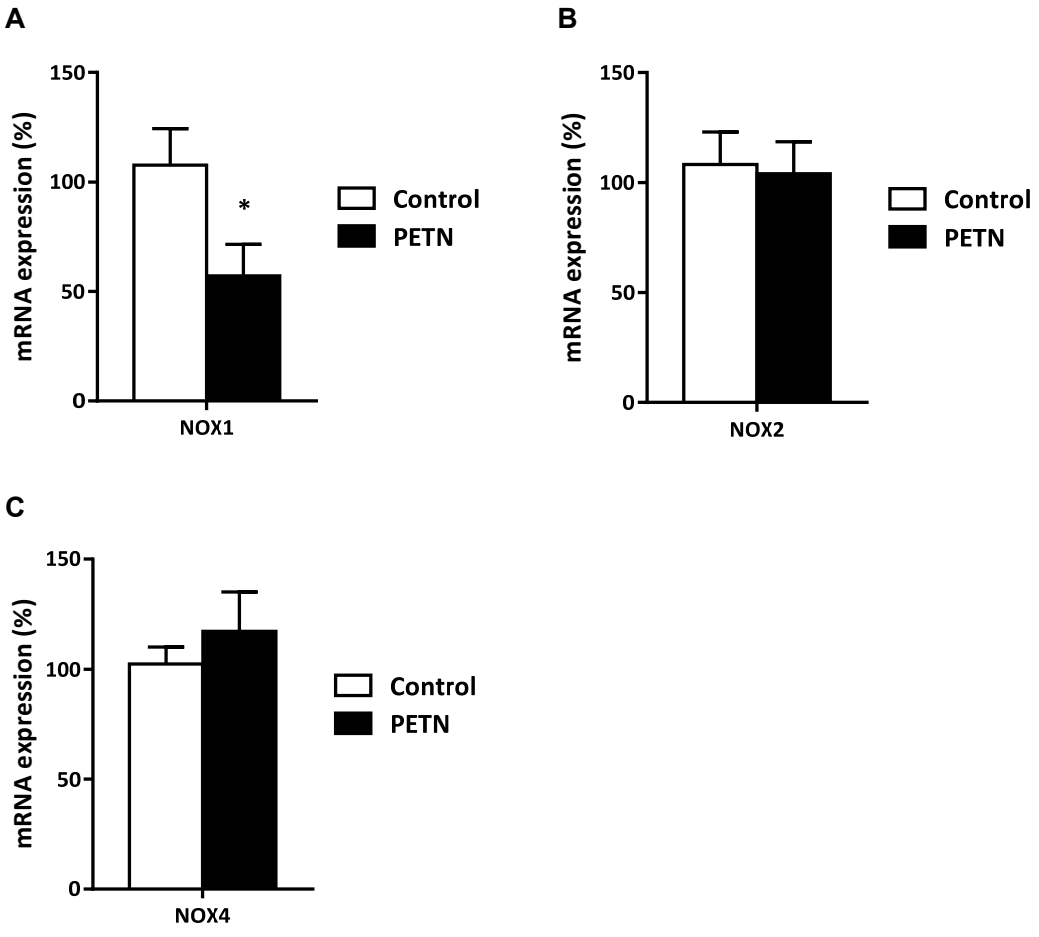

Figure 16: Effect of maternal PETN treatment on NADPH oxidases in female F1 rats.

The aorta of 8-month-old female F1 rats was isolated and the mRNA expression of NADPH oxidases (NOX1, NOX2, NOX4) was analyzed with qRT-PCR (n=11 in Control group; n=8 in PETN group). Data represent mean±SEM. * P <0.05, compared with Control.

4.9 Effect of maternal PETN treatment on SIRT1 in F1 rats

Sirtuin 1 (SIRT1) is a member of the sirtuin family. It is encoded by the SIRT1 gene. SIRT1 upregulates eNOS activity and inhibits endothelial cell senescence, and reduced SIRT1 is related to oxidative stress and reduced NO-dependent dilation (Davis et al., 2013).

In the aorta of 8-month-old female F1 rats, the mRNA expression of SIRT1 was increased significantly in the PETN group (Figure 17). This result was confirmed by Western blot experiment, which showed an enhanced SIRT1 protein level in the PETN group (Figure 17).

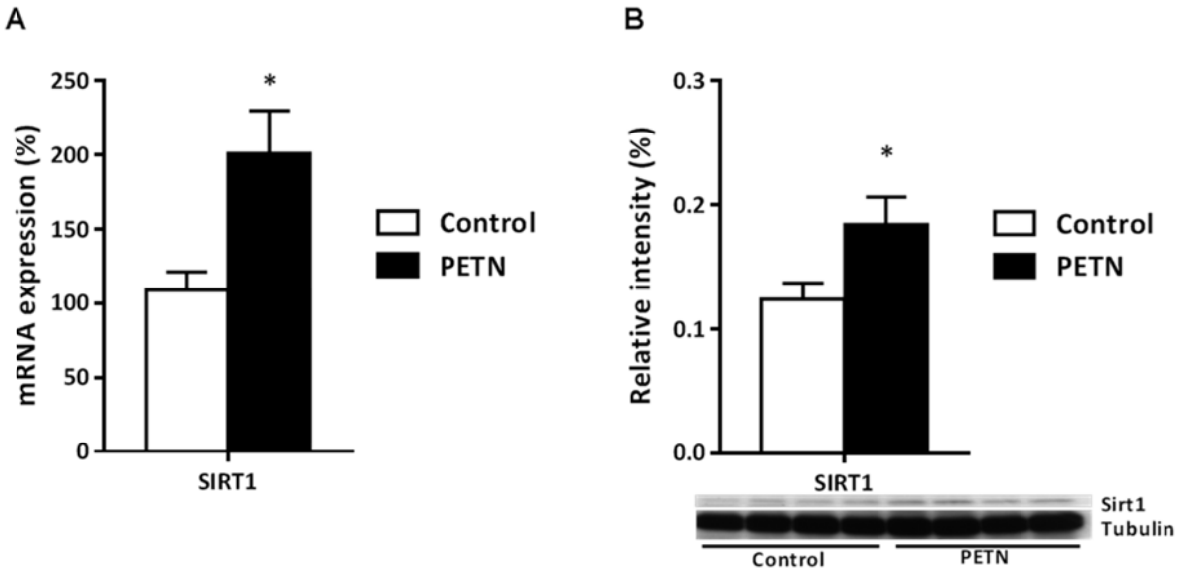

Figure 17: Effect of maternal PETN treatment on SIRT1 in female F1 rats.

The aorta of 8-month-old female F1 rats was isolated and the mRNA expression of SIRT1 was analyzed with qRT-PCR (n=11 in Control group; n=8 in PETN group). Data represent mean±SEM. * P <0.05, compared with Control.

5 Discussion

Essential hypertension is a major risk factor for the development of cardiovascular disease (CVD). Patients with essential hypertension have structural alterations of the precapillary arterioles that result from incompletely understood mechanisms triggered by baro-mechanical stress and impaired endothelial function (Rodriguez-Iturbe, 2006). Essential hypertension is associated with an activation of the circulating and local renin-angiotensin system and increased oxidative stress within the vascular wall (Hamilton, 2002, Touyz, 2003). Patients with essential hypertension need life-long anti-hypertensive treatment.

The discovery of “perinatal programming” put a new sight on the treatment of essential hypertension. The conditions in utero and early postnatal life play a role in a predisposition to adult hypertension (Nuyt, 2008). PETN is an organic nitrate; it is used for prevention of angina pectoris in clinic. PETN has the ability of activating gene expression networks that result in cardio-protection (Pautz et al., 2009). Moreover, the compound improves endothelial dysfunction, inhibits NADPH oxidase activity, inhibits mitochondrial superoxide production, and prevents eNOS uncoupling (Schuhmacher et al., 2010). However, PETN does not lower blood pressure when applied directly (Kristek et al., 2003). The present study is the first to test the therapeutic potential of PETN in “perinatal programming” for the treatment of hypertension. In the following, the effect of maternal PETN treatment on the offspring are discussed.

5.1 Effect of maternal treatment with PETN on blood pressure in parents and offspring SHRs

Direct administration of PETN, an exogenous NO donor, does not decrease blood pressure in SHR (Kristek et al., 2003). The same result was observed in our study. The F0 animals were treated with PETN until the end of lactation. PETN treatment had no effect on the blood pressure development of SHR. In contrast, maternal PETN treatment did decrease blood pressure in offspring, especial female SHR. These

results indicate that a blood pressure reduction in the F0 animal is not necessary for the programming effect of PETN.

The reduction of blood pressure in the female F1 animals was observed in their adult life, from 4 month old to 8 month old. No significant effect was seen in the male F1 animals. The mechanisms underlying this gender difference are still unclear and need to be analyzed in future studies.

For the effect in female F1 rats, it is conceivable that maternal PETN treatment modifies the condition in uterine and early post-natal development, which results in persistent changes in the offspring. In the present study, we aimed to find out such changes in the female F1 rats.

5.2 Effect of maternal PETN treatment on vascular constriction in offspring SHR

The general process of vascular constriction occurs by either, or a combination of, electromechanical and pharmacomechanical coupling. The former refers to stimuli directly initiating a change in membrane potential (for example, application of KCl or cell stretch), while the latter largely refers to receptor-mediated stimuli that involve activation of trimeric G-protein signaling and generation of second messengers (Hill and Meininger, 2012). There are several kinds of receptors involved in vasoconstriction, including the α1 adrenergic receptor, angiotensin II type 1 receptor, and the endothelin ET_A receptor.

5.2.1 Effect on α1 adrenergic receptors

Most blood vessels (arteries and veins) in the body are innervated by sympathetic adrenergic nerves, which release norepinephrine as a neurotransmitter. Norepinephrine preferentially reacts with α1, which results in increased intracellular Ca^{2+} and then smooth muscle contraction. α1a, α1b and α1d are the subtypes of α1 adrenergic receptors. The function of α1b has been clarified by the use of knockout

technology. In aorta from α1b-knockout mice there is a small reduction in the potency of norepinephrine as compared to wild type (Cavalli et al., 1997), indicating a minor contractile role of α1b in mouse aorta (Daly et al., 2002). The α1d adrenergic receptor predominantly mediates contractions with some regulatory role for the α1b adrenergic receptor (Deighan et al., 2005). Mouse aortic contractions to norepinephrine are unaffected by α1b knockout, markedly reduced by α1d knockout, but abolished by combination of α1b/α1d knockout, suggesting α1b and α1d subtypes participate cooperatively in blood pressure regulation (Docherty, 2010, Hosoda et al., 2005). In our study, α1b was significantly downregulated in mRNA level, while α1d was significantly upregulated at mRNA level. The contractile response to norepinephrine, however, did not change in the aorta of PETN offspring. If the vasoconstriction is potentially compensated by α1a, which also predominantly mediates contraction to norepinephrine (Aboud et al., 1993), is currently unknown. Nevertheless, our results indicate that the blood pressure reduction in female F1 SHR cannot be explained by the changes in α1 adrenergic receptors.

5.2.2 Effect on RAS components

Angiotensin II receptor type 1 (Agtr1) mediates vasoconstriction, thirst and release of vasopressin and aldosterone, fibrosis, cellular growth and migration whereas angiotensin II receptor type 2 (Agtr2) mediates vasodilation, NO release and usually inhibition of growth (Fyhrquist and Saijonmaa, 2008). Agtr1 and Agtr2 genes are reportedly associated with hypertension, but they are also inconsistently associated with response to anti-hypertensive therapy (Frazier et al., 2004, Hingorani et al., 1995, Kurland et al., 2001, Miller et al., 1999). In the present study, Agtr1a and Agtr2 mRNA expression were not changed by maternal PETN treatment. To verify our findings, the vascular reactivity was done with exogenous Ang II. No difference in vasoconstriction to Ang II was found between control group and PETN group.

Angiotensin-converting enzyme (ACE) is a dipeptidyl carboxypeptidase that converts Ang I into Ang II. Whereas angiotensin-converting enzyme 2 (ACE2) is a carboxypeptidase which cleaves one residue from Ang I to generate angiotensin 1-9

and a single residue from Ang II to generate Ang 1-7 (Fyhrquist and Saijonmaa, 2008). ACE2 inactivates angiotensin II and is a negative regulator of the system. In the present study, no significant changes in the mRNA expression of ACE or ACE2 were found. Collectively, these results indicate that the blood pressure reduction in the female F1 animals is not mediated by changes of the RAS components.

5.2.3 Effect on endothelin receptor

Endothelin-1 is a potent endothelium-derived vasoconstrictor. In endothelial cells, prepro ET and big ET are cleaved by ET converting enzymes into isoforms ET-1, 2 and 3. These ET isoforms bind with different affinities to ET_A and ET_B receptors in vascular smooth muscle (VSM), and in turn increase [Ca^{2+}], protein kinase C and mitogen-activated protein kinase and other signaling pathways of VSM contraction and cell proliferation (Khalil, 2011). ET_AR mediates vascular smooth muscle (VSM) contraction. ET_BR in the endothelium mediates the release of relaxing factors such as NO, prostacyclin and endothelium-derived hyperpolarizing factor, and could also play a role in ET-1 clearance (Mazzuca and Khalil, 2012). An increase in the expression of ET_AR or a decrease in the expression of ET_BR is expected to cause an increase in blood pressure and hypertension. In the present study, an increase in ET_AR mRNA was observed in the aorta of female F1 animals of the PETN group. The vasoconstriction response to exogenous ET-1 was slightly, but not significantly, enhanced. Thus, the blood pressure reduction in the female F1 animals in response to maternal PETN treatment cannot be explained by changes in the endothelin system.

5.3 Effect of maternal PETN treatment on vascular dilation in offspring SHRs

Some blood vessels are innervated by parasympathetic cholinergic or sympathetic cholinergic nerves, both of which release acetylcholine as their primary neurotransmitter. Acetylcholine can induce vasodilation by several mechanisms, including NO and prostaglandin production. The NO production is a major

endogenous vasodilator system made by endothelium, counterbalancing the vasoconstriction produced by the sympathetic nervous system and RAS.

In the present study, exogenous acetylcholine was used as a tool to test endothelial function. The acetylcholine-induced vasodilation was better in the PETN group, indicating that maternal PETN treatment improves endothelial function in the offspring. Previous studies have shown that direct administration of PENT improves endothelium-dependent vasodilation in SHR (Dovinova et al., 2009) or in Ang II-treated rats (Schuhmacher et al., 2010). Our study is the first to show the effect of maternal PETN treatment.

In addition to NO, endothelial cells also produce vasoactive prostanoids, and endothelium-derived hyperpolarizing factor (EDHF). In our study, the acetylcholine-induced vasodilation could be largely blocked by L-NAME, a NO synthase inhibitor, indicating that NO plays a major role in our study setting. The signal cascade downstream of NO is likely to remain unchanged, because the vasodilator response to SNP, an exogenous NO donor, was not changed. Collectively, these results indicate that the improvement of endothelial function by maternal PETN treatment implicates enhanced endothelial NO production.

Multiple mechanisms could contribute to an increased endothelial NO production: increased eNOS expression/activity, and/or improved eNOS coupling. In the present study, eNOS mRNA and protein expression were increased in the female F1 SHR. This is one mechanism contributing to the enhanced endothelial NO production.

Prevention of eNOS uncoupling could represent another potential mechanism. Hypertension, including SHR, is associated with eNOS uncoupling (Li et al., 2013).. eNOS uncoupling refers to the phenomenon that eNOS can be converted from an NO-producing enzyme to a molecule that generates superoxide under pathological conditions. A number of mechanisms are implicated in eNOS uncoupling, with deficiency of the NOS cofactor tetrahydrobiopterin (BH_4) representing the major cause for eNOS uncoupling (Li et al., 2013). A BH_4 deficiency is evident for SHR (Li et al., 2006).

GTP cyclohydrolase 1 (GCH-1) is the first and rate-limiting enzyme in BH_4 biosynthesis. In the present study, we found that GCH-1 mRNA and protein were significantly increased by maternal PETN treatment. Thus, this may improve eNOS coupling and NO production.

5.4 Effect of maternal PETN treatment on redox genes in offspring SHRs

Oxidative stress may play an important role in the reduction of NO bioavailability. 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 activity and by causing eNOS uncoupling via oxidation of BH_4 (Schuhmacher et al., 2010, Kuzkaya et al., 2003).

5.4.1 Effect on NOX

In the vascular system, reactive oxygen species (ROS) play a physiological role in controlling endothelial function and vascular tone and a pathophysiological role in inflammation, hypertrophy, proliferation, apoptosis, migration, fibrosis, angiogenesis, and rarefaction, important in vascular remodeling and endothelial dysfunction associated with hypertension (Briones et al., 2011). NADPH oxidases are the major sources of ROS in vascular physiology and pathophysiology. NADPH oxidases are a family of enzymes with each member being distinguished by the specific NOX catalytic subunit. Five isoforms have been discovered in mammals (NOX1 to NOX5; NOX5 not found in rodents) (Cave et al., 2006).

Greater mRNA expression of NOX1, NOX2, and NOX4 were found in aorta and mesenteric artery from SHR versus normotensive Wistar-Kyoto rats, contributing to oxidative stress in SHR (Li et al., 2006, Briones et al., 2011). In the present study, the mRNA expression of NOX1 was significantly downregulated by maternal PETN treatment. NOX1 may be responsible for superoxide production and redox signaling in pathological conditions such as hypertension (Chose et al., 2008). Thus, the observed

NOX1 downregulation by maternal PETN treatment may contribute to a reduction of oxidative stress in SHR.

5.4.2 Effect on anti-oxidant enzymes

In the present study, we found an upregulation of anti-oxidant enzymes, including HO-1, SOD2 and GPx1, in the aorta of female F1 SHR in response to maternal PETN treatment.

Some previous studies have shown an effect of direct PETN administration on HO-1. In contrast to nitroglycerin, PETN did not induce nitrate tolerance. Treatment of Wistar rats with PETN led to upregulation of vascular HO-1. Inhibition of HO-1 expression by apigenin induced "tolerance" to PETN (Wenzel et al., 2007). In the mouse model of angiotensin II-induced hypertension, PETN treatment improved endothelial dysfunction. Haploinsufficiency of HO-1 completely abolished the beneficial effects of PETN in angiotensin II-treated mice (Schuhmacher et al., 2010). These results indicate that HO-1 is a crucial mediator of the beneficial effects of direct PETN treatment. The HO-1 upregulation found in the female F1 rats in response to maternal PETN treatment may thus be of great importance.

GPx1 reduces fatty acid hydroperoxides and H_2O_2 thereby protecting cells against oxidative damage. Reduction in GPx1 causes endothelial dysfunction and subsequent vascular remodeling (de Haan and Cooper, 2011). In the present study, maternal PETN treatment led to upregulation of GPx1 mRNA and protein expression in the aorta of female offspring. This may contribute to a reduction in vascular oxidative stress.

SOD enzymes reduce oxidative stress by catalyzing the dismutation of superoxide radical anions. SOD2 is a key regulator of oxidative stress and endothelial function. Heterozygous SOD2 deficient (SOD2+/-) mice develop hypertension with age (Fukai and Ushio-Fukai, 2011). Transgenic mice overexpressing SOD2 demonstrated attenuated Ang II-induced hypertension and vascular oxidative stress (Dikalova et al., 2010). Therefore, the upregulation of SOD2 we observed in the female F1 rats in

response to maternal PETN treatment may represent a mechanism contributing to reduction of oxidative stress.

In addition, we have observed an upregulation of SIRT1 in the aorta of female F1 SHR. SIRT1 may on one hand reduce oxidative stress by upregulation of antioxidant enzymes, including GPx1 and SOD2 (Li et al., 2012, Xia et al., 2010). On the other hand, SIRT1 enhances eNOS expression (Xia et al., 2013) and stimulates eNOS activity by deacetylating eNOS lysine residues in the calmodulin-binding domain (Li et al., 2012, Mattagajasingh et al., 2007).

In conclusion, maternal PENT treatment leads to blood pressure reduction in the female offspring. Upregulation of anti-oxidant enzymes, downregulation of NOX1, and enhanced endothelial NO production may represent molecular mechanisms contributing to the effect (Figure 18). It is noteworthy that the changes of gene expression were (still) evident at the age of 8 months. It is conceivable that epigenetic mechanisms are involved in such a persistent effect on gene regulation. This is currently being investigated in our laboratory.

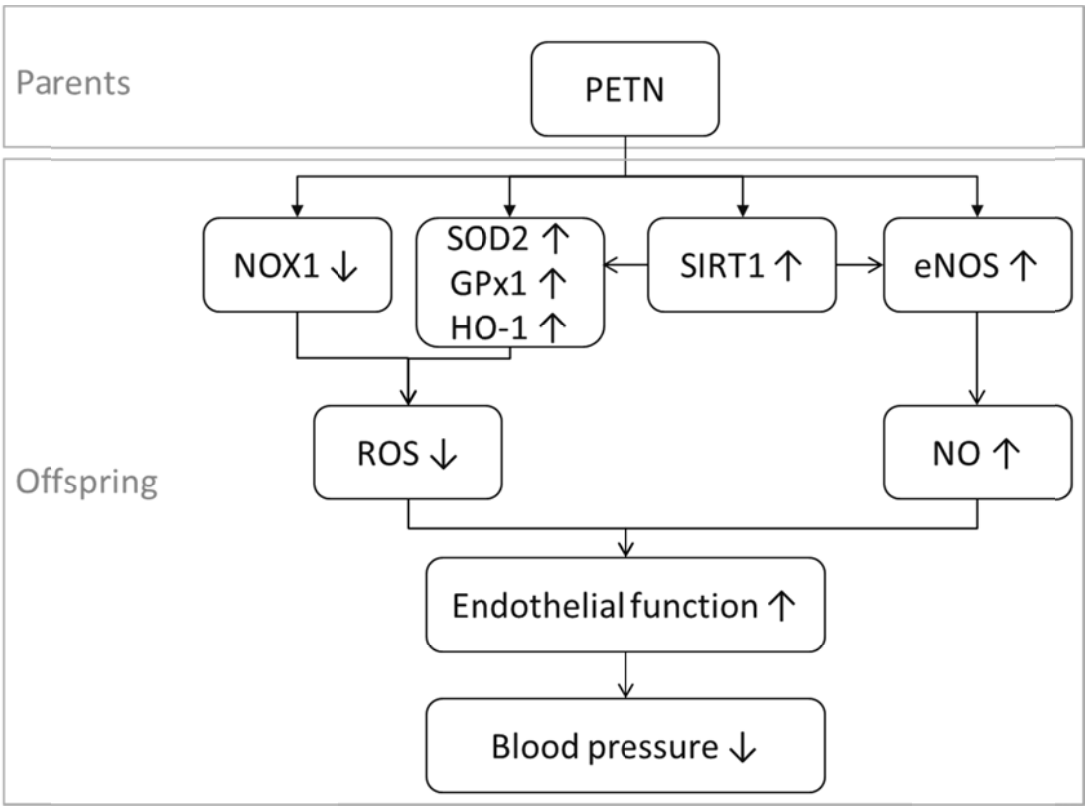

Figure 18: Postulated mechanisms of maternal PETN treatment.

6 Summary

Pentaerithrityl tetranitrate (PETN) is an organic nitrate used for prevention of angina pectoris in clinic. PETN has little effect on blood pressure when administrated directly. The present study was designed to test the “perinatal programming” effect of PETN in spontaneously hypertensive rats, a rat model of genetic hypertension.

The F0 parent SHR animals were treated with PETN (50 mg/kg/day) during pregnancy and lactation period; the offspring received standard chow after weaning. Blood pressure was measured in the offspring at ages of 3 to 8 months.

Maternal PETN treatment had little effect on blood pressure in male offspring. In the female F1 SHR animals, however, a persistent reduction in blood pressure was observed. The systolic blood pressure in the female F1 rats of the PETN group was about 13 mmHg lower at the age of 4 months, and was still 10 mmHg lower than that of control group at the age of 8 months. This long-lasting effect of maternal PETN treatment on blood pressure was accompanied by a substantial change in gene expression even evident at the age of 8 months. In the aorta of female F1 SHR animals, changes were found in the expression of α adrenergic receptors and endothelin receptors with minor functional contribution. In contrast, a clear role was evident for nitric oxide (NO). Maternal PETN treatment led to an upregulation of the endothelial NO synthase (eNOS) and the GTP cyclohydrolase I (GCH-1). The latter is crucial for tetrahydrobiopterin (essential eNOS cofactor) biosynthesis and thus for eNOS functionality. In addition, the anti-oxidant enzymes, including the mitochondrial superoxide dismutase (SOD2), the glutathione peroxidase I (GPx1) and the heme oxygenase-1 (HO-1), were upregulated, and the superoxide-producing enzyme NADPH oxidase NOX1 was downregulated. This may reduce oxidative stress and improve NO bioavailability. Finally, sirtuin 1 (SIRT1) was upregulated by maternal PETN treatment which may represent additional regulation mechanism for SOD2, GPx1 and eNOS. In organ chamber experiment, the acetylcholine-induced, endothelium-dependent vasodilation was enhanced in aorta from female F1 SHR animal of the PETN group. This improved endothelial function, which may result from

expression changes of the abovementioned genes, is likely to represent a key mechanism for the blood pressure reduction in the offspring.

7 Zusammenfassung

Pentaerithrityltetranitrat (PETN) ist ein organisches Nitrat und wird in der Klinik zur Behandlung der Angina Pectoris eingesetzt. PETN hat, wenn direkt verabreicht, kaum Wirkung auf den Blutdruck. Diese Arbeit wurde konzipiert, um einen potentiellen „perinatalen Programmierung“-Effekt von PETN in spontan-hypertensiven Ratten (SHR), einem Rattenmodel der genetischen Hypertonie, zu testen.

Die F0-Elterntiere wurden mit PETN (50 mg/kg/Tag) während der Schwangerschaft und der Laktation behandelt; die F1-Nachkommen bekamen nach der Ablaktation normales Haltungsfutter. Der Blutdruck wurde an den Nachkommen vom 3. Monat bis zum 8. Monat nach der Geburt gemessen.

Maternale PETN-Behandlung hatte kaum Wirkung auf den Blutdruck in den männlichen SHR-Nachkommen. Dagegen zeigten die weiblichen Nachkommen der PETN-Behandlungsgruppe eine persistente Reduktion des Blutdrucks. Der systolische Blutdruck war in den weiblichen Nachkommen in der PETN-Gruppe etwa 13 mmHg niedriger im 4. Monat und etwa 10 mmHg niedriger im 8. Monat als in den Kontrolltieren. Dieser lang-anhaltende Effekt ging mit einer substanziellen Änderung der Genexpression einher, die auch beim 8. Monat noch nachzuweisen war. In den Aorten der weiblichen F1-Nachkommen wurde Veränderungen an Genexpression der α-adrenergen Rezeptoren sowie Endothelin-Rezeptoren festgestellt, die aber funktionell von minimaler Bedeutung für die PETN-Wirkung waren. Hingegen war eine klare Rolle des StickstoffmoNOXid (NO) zu sehen. Maternale PETN-Behandlung führte zur Heraufregulation der endothelialen NO-Synthase (eNOS) und der GTP-Cyclohydrolase I (GCH-1). GCH-1 ist für die Biosynthese des Tetrahydrobiopterins, eines essentiellen eNOS-Kofaktors, entscheidend, und dadurch auch für die eNOS-Funktionalität. Zusätzlich wurden auch anti-oxidative Enzyme wie die mitochondriale Superoxid-Dismutase (SOD2), die Glutathion-Peroxidase 1 (GPx1) und die Hem-Oxygenase 1 (HO-1) heraufreguliert, und die Superoxid-produzierende NADPH-Oxidase NOX1 herunterreguliert. Dies kann zur Verminderung vom oxidativen Stress und Erhöhung der NO-Bioverfügbarkeit führen. Letztlich wurde auch

die Sirtuin 1 (SIRT1) durch maternale PETN-Behandlung heraufreguliert, die auch zur Heraufregulation der SOD2, GPx1 und eNOS beitragen kann. Im Organbad-Experiment wurde die Acetylcholin-induzierte, Endothel-abhängige Vasodilatation in der Aorta der weiblichen Nachkommen der PETN-Gruppe verstärkt. Diese verbesserte Endothelfunktion, was vermutlich aus der Genexpressionsänderung resultiert, stellt sehr wahrscheinlich einen Schlüsselmechanismus der Blutdrucksenkung in den Nachkommen der PETN-behandelten F0-Tiere dar.

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