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THE PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR GAMMA AGONIST PIOGLITAZONE IMPROVES NITRIC OXIDE AVAILABILITY, RENIN-ANGIOTENSIN SYSTEM AND ABERRANT REDOX REGULATION IN THE KIDNEY OF PRE-HYPERTENSIVE RATS

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The peroxisome proliferator-activated receptor gamma (PPARγ) is a ligand-dependent nuclear receptor. It plays an important role in kidney physiology, where it might contribute to arterial blood pressure regulation and hypertension development by modulation of several signaling pathways. In our study we focused on the effect of PPARγ agonist pioglitazone on changes in the nitric oxide synthase (NOS) expression and activity, the renin-angiotensin system (RAS) cascade, and redox homeostasis signaling pathways in the renal cortex of young pre hypertensive rat models. Young (5-weeks old) spontaneously hypertensive (SHR) and borderline hypertensive (BHR) rats were treated by pioglitazone (PIO, 10 mg/kg/day) during 10 days. Blood pressure (BP) was determined by plethysmography method. Changes in lipid profile were detected in plasma with standard kits using biochemical analyser. Gene expression has been detected by qRT-PCR and protein level was determined using Western blot analysis. Superoxide dismutase (SOD) and catalase (CAT) activities were determined spectrophotometrically and the total enzyme activity of NOS was measured using a radioactive assay based on conversion of [^{3}H] L-arginine to [^{3}H] L- citrulline. Administration of pioglitazone decreased BP in BHR and slowed down the development of BP increase in young SHR animals. For NOS, activation by PPARγ correlated with increase in gene and protein expression of NOS isoforms and in total enzyme activity both in BHR and SHR. In the AT1R/Nox pathway, the treatment did not significantly influence mRNA expression of the p22phox subunit of NADPH oxidase (Nox) and AT1R, but up-regulated the 'pro-vasodilatatory' Mas and AT2R receptors in both BHR and SHR groups. Pioglitazone treatment affected redox regulation. Increase in gene expression of nuclear factor E2-related factor 2 (Nrf2) and SOD isoforms correlated with SOD and CAT enzyme activities. The group treatment-to-control ratios, BHR Pioglitazone to BHR control and SHR Pioglitazone to SHR control for gene expression increased by 10% to 230%. The largest effect of PPARγ has been observed in SOD1, SOD3 and the Mas receptor gene treatment-to-control ratios. The most prominent differences between BHR and SHR were observed in SOD1 and Mas receptor expressions, with large effects of opposite sign in BHR versus SHR. Our data indicate that an increase of NO release activates signaling in the renal cortex of pre-hypertensive rats after pioglitazone treatment. Improvement of NO availability, AT2R, Mas receptors and aberrant redox regulation is thought to be the major correlated mechanisms mediating the BP decrease affected by the PPARγ agonist treatment. We also observed that the most sensitive tissue responses to PPARγ-dependent activation of Nrf2 have been primarily found in the kidney of young hypertensive animals.

Key words: *peroxisome proliferator-activated receptor gamma, pioglitazone, signaling pathway, nuclear factor E2-related factor 2, nitric oxide synthase, renin-angiotensin system, superoxide dismutase, catalase, kidney*

INTRODUCTION

The peroxisome proliferator-activated receptor gamma (PPARγ) is a nuclear receptor that plays an important role in kidney physiology. It is a ligand-dependent intracellular orphan nuclear receptor that affects transcription of specific genes and is mainly expressed in the renal medullary collecting duct, though a lower expression was observed also in renal glomeruli and renal microvasculature (1).

PPARγ is a ligand activated transcription factor activated with several agonists such as pioglitazone and rosiglitazone which can improve metabolic syndrome and hypertension (2-4). Activation of PPARγ is involved in the regulation of energy metabolism (5, 6), oxidative stress, antioxidant response and inflammation in the cardiovascular system (5, 7, 8). It can be protective in cardiomyocytes against oxidative damage (9) and also in hypertension development (6, 10). PPARγ is, reportedly, also a regulator of renal hemodynamics and water and sodium

transport in kidney. Several studies indicate that PPARγ is also involved in normal kidney development, renal lipid metabolism, and activation of the renin-angiotensin system (RAS) (11, 12).

PPARγ can modulate redox homeostasis, where the major regulatory system is Keap1/Nrf2/ARE signaling (ARE, antioxidant response element; Keap, Kelch-like ECH associated protein 1; Nrf2, nuclear factor E2-related factor 2). Polvani *et al.* point out that PPARγ can affect redox homeostasis by reciprocal transcriptional regulation between PPARγ and Nrf2. PPARγ agonists might up regulate Nrf2 expression (13, 14) and Nrf2 activation can increase PPARγ expression at both mRNA and protein levels (15).

Elevated production of reactive oxygen species (ROS) in the brain, in the sympathetic nervous system (SNS) (16, 17) and in the kidney (18, 19) was observed in the pathogenesis of hypertension. Increased ROS levels might affect NOS 'redox switches'. These include S-glutathionylation of cysteine residues in the reductase domain, H_2O_2/$ONOO^-$-mediated phosphorylation of endothelial nitric oxide synthase (eNOS), direct oxidative depletion of tetrahydrobiopterin, and oxidative disruption of the zinc-sulphur complex in the dimer binding interface (20). Treatment by thiazolidinediones (TZDs) decreases ROS production in vascular smooth muscle cells (21, 22) and endothelial cells (21, 23) allowing a decrease in BP. Chan *et al.* noticed that RSG administration has a central antihypertensive effect. That can be mediated through PPARγ-dependent transcriptional up-regulation of antioxidant mechanisms (2). Furthermore, Nrf2 activation increased eNOS phosphorylation at Ser^{1177} and improved endothelial function by different pathways. Nrf2 might promote gene expression for antioxidant molecules that counter the ROS-induced impairment of endothelial function and eNOS uncoupling (15).

The ligand-dependent activation of PPARγ influences the insulin signaling pathway by modulation of expression and/or phosphorylation of specific signaling molecules, resulting in reestablishment of the balance between vasodilatation and vasoconstriction effects due to insulin receptor activation. Administration of RSG increases vascular PPARγ expression, resulting in restoration of PI3K/Akt/eNOS signaling activation, followed by improvement of endothelial function due to increased NO release (24, 25). In the study of Li *et al.*, results suggest that administration of TZDs to young and adult normotensive Wistar Kyoto rats has no influence on vascular insulin resistance, measured as the function of isolated aortic vasodilatory response to insulin *in vitro*, and the administration did not change protein expression of the PI3K/Akt/eNOS signaling cascade (25).

Administration of TZDs improve insulin sensitivity and decreases BP in non-diabetic hypertensive patients (26) as well as in diabetic patients (27).

Another important mechanism, which plays an essential role in cardiovascular and renal physiology, is the renin-angiotensin system (RAS system). Deregulation of RAS contributes not only to hypertension development but also to cardiovascular complications such as atherosclerosis, ischemic heart disease and heart failure (28, 29). RAS can be affected by PPARγ activation. PPARγ-dependent mechanisms affecting BP regulation and hypertension development include attenuation of the expression of angiotensin-receptor 1 (AT1R) gene (24, 30, 31), increased endothelial NO synthesis (32) and prevention of oxidative stress and endothelial dysfunction.

Studies on several types of experimental animals and different types or stages of hypertension and cardiovascular diseases observed different responses in redox regulation and cell signaling (2-4). Apparently, the normotensive Wistar-Kyoto rats, the borderline hypertensive rats (BHR), and the spontaneously hypertensive rats (SHR) respond differently (33). Also, it was observed that PPARγ is mainly expressed in kidney, which seems to be a very effective tissue in BP regulation (34). In previous studies we observed influence of main regulatory systems located in the central (brainstem) and peripheral (left ventricle) parts of experimental animals (3, 35). Main intracellular signaling was highly effective in brain stem, where it influenced SOD2 and changes in protein levels of Akt kinase and β-catenin. In a metabolic syndrome model, pioglitazone improved nNOS levels and antioxidant regulation in rostral ventrolateral medulla (4).

The present study uses a PPARγ agonist, pioglitazone, to shed light on PPARγ activation related redox regulation and cell signaling in hypertension. Two models of hypertension development, young BHR and SHR rats, were used. Since PPARγ is mainly expressed in the kidney, we focused on the effect of PPARγ agonist pioglitazone on the signaling pathways in the renal cortex of young pre-hypertensive rats. We studied the signaling pathways of NOS, RAS and Nrf2 activation, and antioxidant responses.

MATERIALS AND METHODS

Experimental design: animal model and treatment protocol

In this study, young (5-weeks old) male borderline hypertensive rats (BHR; offspring of spontaneously hypertensive females and Wistar-Kyoto males) and spontaneously hypertensive rats (SHR) were used. All rats were born in our certified animal facility (Institute of Normal and Pathological Physiology, SAS) to maintain the same environmental background for all animals. The rats were allowed at least one week to acclimatize to the lab conditions, housed three per cage at constant temperature 22 – 24ºC and humidity (45 – 60%) with a 12/12 h light/dark cycle (light on at 6 a.m. and off at 6 p.m.) and fed a standard pellet diet with tap water *ad libitum*. The BHR and SHR animals were divided into two groups: control group treated with saline solution (Control; n = 6), and a group treated with pioglitazone (Pioglitazone; n = 7). Pioglitazone in saline solution was administered by oral gavage, in the dose of 10 mg/kg/day during 10 days (36). The daily food and water intake, body weight (BW) and BP were measured before, during (in three-day intervals), and after the treatment period. At the end of the experiment, the animals were exposed to CO_2 anesthesia and sacrificed. Whole blood was collected into heparin and Na_2-EDTA solution and kidneys were rapidly excised.

All procedures were performed based on and in accordance with Ethical Committee approval according to the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes, Directive 2010/63/EU of the European Parliament. Experiments were approved by the Department of Animal Wellness, State Veterinary and Food Administration of the Slovak Republic, and in accordance with the guidelines of the Animal Research and Care Committee of the Institute of Normal and Pathological Physiology of the Slovak Academy of Sciences.

Blood pressure determination

Systolic BP was measured non-invasively by tail cuff plethysmography using Statham Pressure Transducer P23XL (Hugo Sachs, Germany). In all groups of rats (Control group and Pioglitazone-treated group of BHR and SHR), blood pressure was measured in the morning between 08:00 a.m. and 11:00 a.m. as described in detail previously (36). Measurements were performed before (day 0), during (days 3 and 6), and after the

treatment period (day 10). Each value was calculated as average of seven measurements.

Sample collection and preparation

At the end of the experiment, the animals were sacrificed and tissues (left kidney, heart) were rapidly excised. Wet mass of the left heart ventricle (LV) was determined to calculate relative weight (LV/BW). Excised kidneys were weighed and weights were registered. Further processing of tissue samples depended on the specific assay. For measurements of total NOS and SOD activities, the tissue samples were cooled in Tris-HCl (pH = 7.4) supplemented with a protease inhibitor cocktail and further processed according to individual methods. The tissue samples for molecular biological (RT-qPCR) and biochemical (Western blot) analyses were frozen in liquid nitrogen and stored at –80ºC until further use. Plasma samples were withdrawn from each rat after 12 h fasting and prepared from whole blood collected into heparin solution. After centrifugation for 5 min at 1200 g, the plasma was collected. Prepared plasma samples were immediately stored at -80ºC until used in the following biochemical assays.

Glycemia and lipid profile determination

Glycemia and lipid profile (cholesterol, LDL, HDL, TAG) were determined in blood plasma at the end of treatment. All parameters were measured by Abbott diagnostics (FH Trencin, Slovakia) using the Architect c16000 biochemical multi-analyser (Abbott, USA). Results are expressed as mean ± SEM and they were analysed by ANOVA followed by Bonferroni-corrected pairwise t-tests.

Gene expression determination

Expression levels of selected genes were determined by real-time quantitative polymerase chain reaction (RT-qPCR). Total RNA from the renal cortex samples was isolated with a TRIsure reagent (Bioline) according to manufacturer's protocol. The isolated total RNA was quantified spectrophotometrically at 260 nm using a Nanodrop 2000 UV-VIS spectrophotometer (Thermo Scientific, USA). The purity of RNA was measured at 260/280 nm (rate ~2.0) and 260/230 nm (rate range: 1.8 – 2.2) for elimination of protein, phenol or other contaminants. The reverse transcription reaction was performed using SensiFAST™ cDNA Synthesis Kit (Bioline, UK) on a Mastercycler Personal (Eppendorf, Germany) according to manufacturer's protocol (1 µg of total RNA per 20 µl of reaction medium). RT-qPCR by amplification of cDNA was performed on a CFX96 Real-Time PCR detection system (BioRad, USA) using the SensiFAST SYBR No ROX kit (Bioline, UK). PCR reaction for each sample was carried out in duplicate for all cDNA. Samples were measured using BioRad CFX Manager Software (version 2.0) and β-actin was used as the housekeeping gene. The primer pair specifications used to amplify the genes studied (AT1R, AT2R, eNOS, Mas, nNOS, Nrf2, p22phox, PPARγ, SOD1, SOD2, and SOD3) as well as a housekeeping gene (β-actin) are listed in Data Sheets. All chemicals used in this study were purchased from SigmaAldrich (Germany) and Merck Chemicals (Germany), unless stated otherwise.

Total catalase activity determination

Total enzyme activity of catalase (CAT) was determined in the 1% tissue homogenates of the renal cortex in phosphate buffer (50 mM, pH = 7.4) using a yellowish product of reaction between undecomposed hydrogen peroxide (20 mM) and ammonium molybdate (37). The hydrogen peroxide solution was freshly diluted and standardized daily using a molar extinction coefficient of 43.6 $M^{-1}.cm^{-1}$ at 240 nm. The CAT activity was determined at 374 nm using a Nanodrop 2000 UV-VIS (Thermo Scientific, USA) and expressed as U/mg of tissue proteins as determined using the Lowry method.

Total nitric oxide synthase activity determination

Total enzyme activity of NOS was determined in 20% tissue homogenates of the renal cortex using measurement of the conversion of radioactive [^{3}H]-L-arginine (MP Biomedicals, USA) to [^{3}H]-L-citrulline as described previously (36). The product was detected by a Tri-Carb 2910TR liquid scintillator (PerkinElmer, USA) and expressed as pmol/min/mg of tissue proteins as determined using the Lowry method.

Total superoxide dismutase activity determination

Total enzyme activity of SOD was analysed using the SOD determination kit (Sigma-Aldrich, Germany) in 0.5% tissue homogenates of the renal cortex samples. The rate of WST-1 reduction with superoxide is linearly related to the xanthine oxidase activity, and is inhibited by SOD. SOD activity was determined as an inhibition activity by measuring the colour decrease of WST-1-formazan production at 450 nm using a microplate reader (Thermo Scientific Multiscan FC, USA) and expressed as U/mg of tissue proteins as determined using the Lowry method.

Electrophoresis and immunochemical Western blot analysis

Samples of denatured protein fractions containing equivalent amounts of proteins per lane (20 µg per lane) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoretic separation, proteins were transferred to a nitrocellulose membrane. The quality of the transfer was controlled by Ponceau S staining of nitrocellulose membranes. Specific anti-eNOS, anti-nNOS (Bioss, USA), anti-Akt kinase and anti-GAPDH as a housekeeper (Santa Cruz Biotechnology, USA)) (incubation at 4ºC overnight) were used for the primary immunodetection. Peroxidase-labelled anti-rabbit immunoglobulin (Cell Signaling Technology, USA) was used as the secondary antibody. Bound antibodies were detected by enhanced chemiluminescence (ECL) detection method. Quantification of the proteins from Western blot records was done using Kodak-In-Vivo FXPro Multispectral System using the Carestream MI-SE software (Kodak, USA). Equivalent protein loading and transfer efficiency were verified by staining for GAPDH.

Statistical analysis

Data are presented as group mean values ± standard error of the mean (SEM) of the number (n) of independent measurements. Analysis of variance one-way ANOVA and unpaired Student's t-test were used for between-groups comparisons in most measurements (biometric parameters, glycemia and lipid profile, gene and protein expression, enzyme activity). Blood pressure measurements were analysed by two-way ANOVA (group × time). ANOVA analyses were followed by a selection of pairwise comparisons using t-tests with Bonferoni correction. Differences were considered significant at $P < 0.05$. GraphPad Prism 5.0 (GraphPad Software Inc., USA) and Statistica 7 (Stat Soft, Inc., USA) were used for the statistical analyses to determine significant differences between groups.

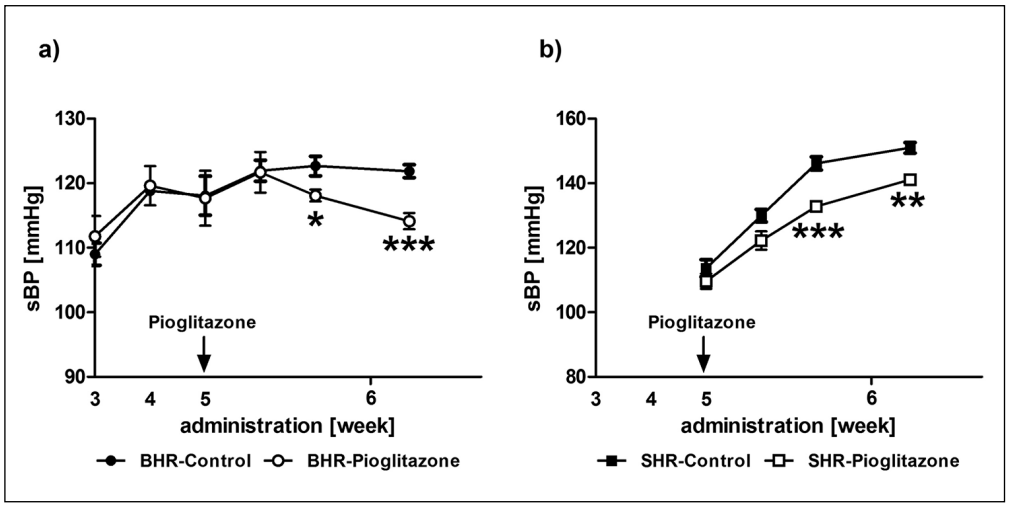

Fig. 1. Effect of pioglitazone on systolic blood pressure in a) BHR and b) SHR. Control groups (BHR-Control; n = 6 and SHR-Control; n = 6), groups treated with pioglitazone (BHR- Pioglitazone; n = 6 and SHR- Pioglitazone; n = 7). Values represent mean ± SEM; asterisks indicate statistical significance compared with phenotype-matched Control group in all tests (*P < 0.05; **P < 0.01, ***P < 0.001).

Table 1. Effect of pioglitazone on basic biometric parameters, glycemia and lipid profile in BHR and SHR. Control groups (BHR-Control; n = 6 and SHR-Control; n = 6), groups treated with pioglitazone (BHR-Pioglitazone; n = 6 and SHR-Pioglitazone; n = 7).
Abbreviations: BW, body weight; HW/BW, heart weight/body weight; LVW/BW, left ventricle weight/body weight; glc, glycemia; CHOL, total cholesterol concentration; HDL, plasma-high density lipoproteins concentration; LDL, plasma-low density lipoproteins concentration; TAG, plasma triglyceride concentration.

	BHR		**SHR**	
	Control	***Pioglitazone***	***Control***	***Pioglitazone***
BW (g)	174.33 ± 2.70	**183.83 ± 1.92***	121.00 ± 2.31	120.57 ± 2.67
HW/BW	3.98 ± 0.05	3.94 ± 0.03	4.00 ± 0.09	4.12 ± 0.05
LVW/BW	2.00 ± 0.07	1.94 ± 0.04	2.28 ± 0.17	2.31 ± 0.08
glc (mmol/l)	5.15 ± 0.24	4.97 ± 0.24	5.94 ± 0.41	5.88 ± 0.20
CHOL (mmol/l)	2.46 ± 0.19	**2.02 ± 0.16****	2.29 ± 0.27	**1.85 ± 0.10***
HDL (mmol/l)	0.78 ± 0.04	**0.65 ± 0.03*****	1.58 ± 0.19	**1.35 ± 0.07****
LDL (mmol/l)	0.20 ± 0.02	**0.17 ± 0.02***	0.47 ± 0.12	**0.35 ± 0.07***
TAG (mmol/l)	1.02 ± 0.23	1.10 ± 0.32	0.88 ± 0.08	0.79 ± 0.06

Values represent mean ± SEM; asterisks indicate statistical significance compared with a phenotype-matched Control group in all tests (*P < 0.05; **P < 0.01; ***P < 0.001).

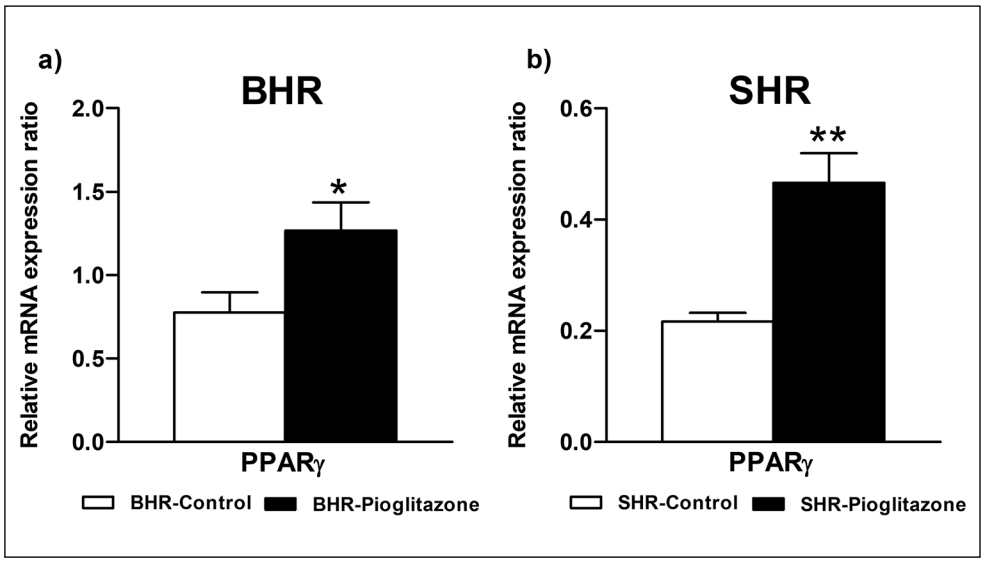

Fig. 2. Effect of pioglitazone on gene expression of PPARγ in a) BHR and b) SHR. Gene expression has been normalized on 'housekeeping' gene β-actin. Control groups (BHR-Control; n = 6 and SHR-Control; n = 6), groups treated with pioglitazone (BHR-Pioglitazone; n = 6 and SHR-Pioglitazone; n = 7). Values represent mean ± SEM; asterisks indicate statistical significance of PIO versus the corresponding Control group (*P < 0.05; **P < 0.01).

RESULTS

Effect of PPARγ agonist pioglitazone on blood pressure, biometric parameters, glycemia, lipid profile and creatinine level

Administration of the PPARγ agonist decreased BP in BHR (*Fig. 1a*) and slowed down the increase of BP in young pre-hypertensive animals (SHR) (*Fig. 1b*).

The effects of pioglitazone treatment on rat body weight, relative weight of the heart and the left ventricle (organ weight/body weight), plasma glucose concentrations and lipid profile were measured and determined (*Table 1*). Administration of pioglitazone slightly increased the BW of BHR. The total cardiac mass and relative weight of heart and LV were not changed significantly in pioglitazone-treated rats. The treatment also did not significantly influence the plasma concentrations of glucose. Pioglitazone administration improved lipid profile and this was associated with a significant reduction of total cholesterol concentration, and lowering of HDL and LDL plasma levels in comparison to control groups (*Table 1*). Plasma triglycerides were not influenced by pioglitazone treatment. Effect of PIO on creatinine level did not show any significant changes in BHR (Control 24.60 ± 5.41 versus PIO 26.50 ± 2.17 μmol/l) and/or SHR animals (Control 24.60 ± 2.09 versus PIO 24.52 ± 2.32 μmol/l).

Effect of pioglitazone on PPARγ activation

Pioglitazone-dependent PPARγ activation was determined using qRT-PCR in the renal cortex after pioglitazone administration. We found that PIO administration markedly elevated PPARγ mRNA levels in both experimental groups (BHR-Pioglitazone, SHR-Pioglitazone) comparing to Control groups (BHR-Control, SHR-Control) (*Fig. 2a* and *2b*).

Effects of PPARγ agonist pioglitazone on endothelial nitric oxide synthase pathway and nitric oxide synthase activities

The eNOS signaling pathway plays an important role in BP regulation. We observed pioglitazone-dependent changes in activation of the NOS signaling pathway in the renal cortex. We found that pioglitazone administration increased gene expression of NOS isoforms. eNOS mRNA and nNOS mRNA

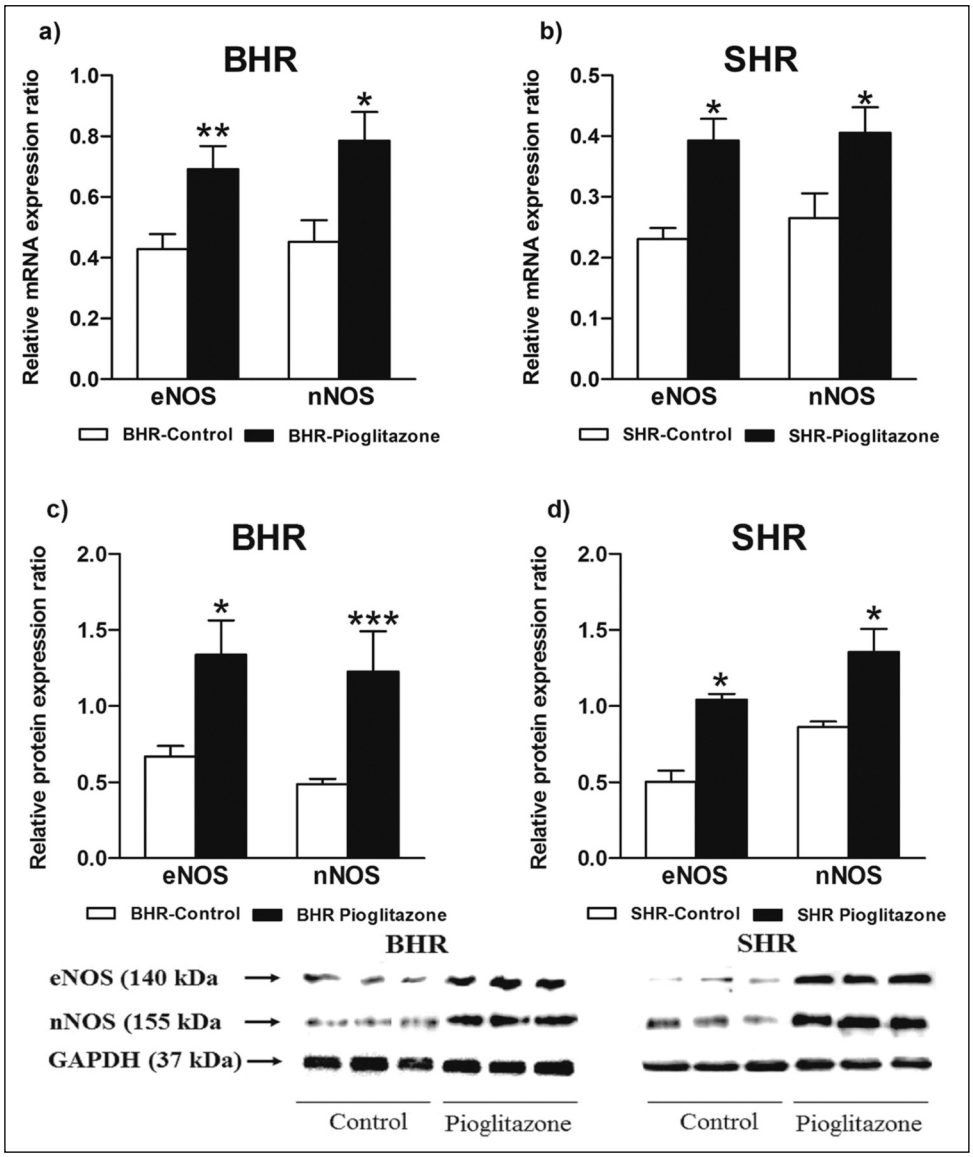

Fig. 3. Effect of pioglitazone on a) gene expression of eNOS and nNOS isoforms in BHR; b) gene expression of eNOS and nNOS isoforms in SHR; c) protein expression of eNOS and nNOS isoforms in BHR and d) protein expression of eNOS and nNOS isoforms in SHR. Gene and protein expression has been normalized on 'housekeeping' gene β-actin or GAPDH. Control groups (BHR-Control; n = 6 and SHR-Control; n = 6), groups treated with pioglitazone (BHR-Pioglitazone; n = 6 and SHR-Pioglitazone; n = 7). Values represent mean ± SEM; asterisks indicate statistical significance of PIO versus the corresponding control group (*P < 0.05; **P < 0.01).

were elevated in BHR (*Fig. 3a*) and similar effects of pioglitazone on eNOS and nNOS mRNA elevation were found in SHR (*Fig. 3b*). Similarly, pioglitazone induced an increase in protein expression of eNOS and nNOS isoforms in both experimental animals (*Fig. 3c* and *3d*). Pioglitazone treatment also significantly increased total enzyme activity of NOS in both BHR (*Fig. 4a*) and SHR (*Fig. 4b*).

Effects of PPARγ *agonist pioglitazone on renin-angiotensin system cascade*

Deregulation of RAS participates in the pathogenesis of hypertension and RAS can be affected by PPARγ activation. We investigated the interaction between pioglitazone dependent activation of PPARγ and the p22phox NADPH oxidase (Nox)

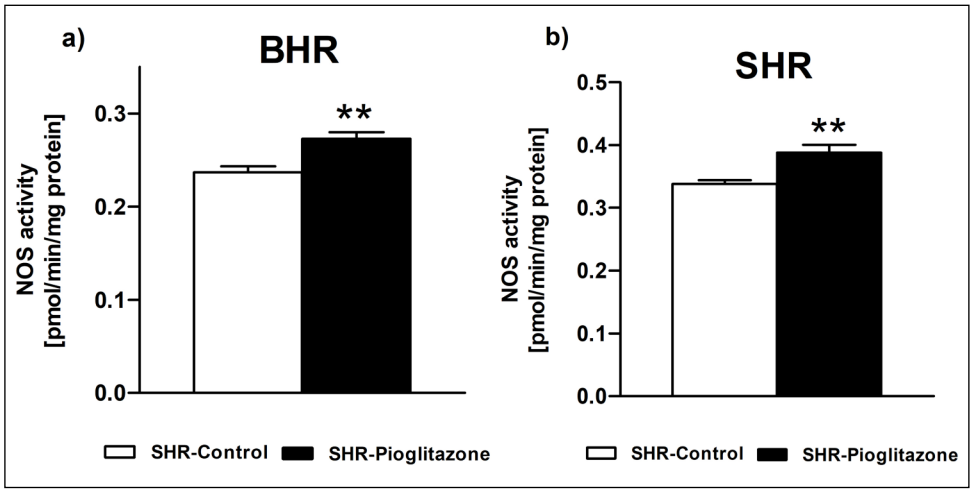

Fig. 4. Effect of pioglitazone on enzyme activities; a) total NOS enzyme activities in BHR and b) total NOS enzyme activities in SHR. Control groups (BHR-Control; n = 6 and SHR-Control; n = 6), groups treated with pioglitazone (BHR-Pioglitazone; n = 6 and SHR-Pioglitazone; n = 7). Values represent mean ± SEM; asterisks indicate statistical significance of Pioglitazone versus the corresponding Control group (*P < 0.05; **P < 0.01).

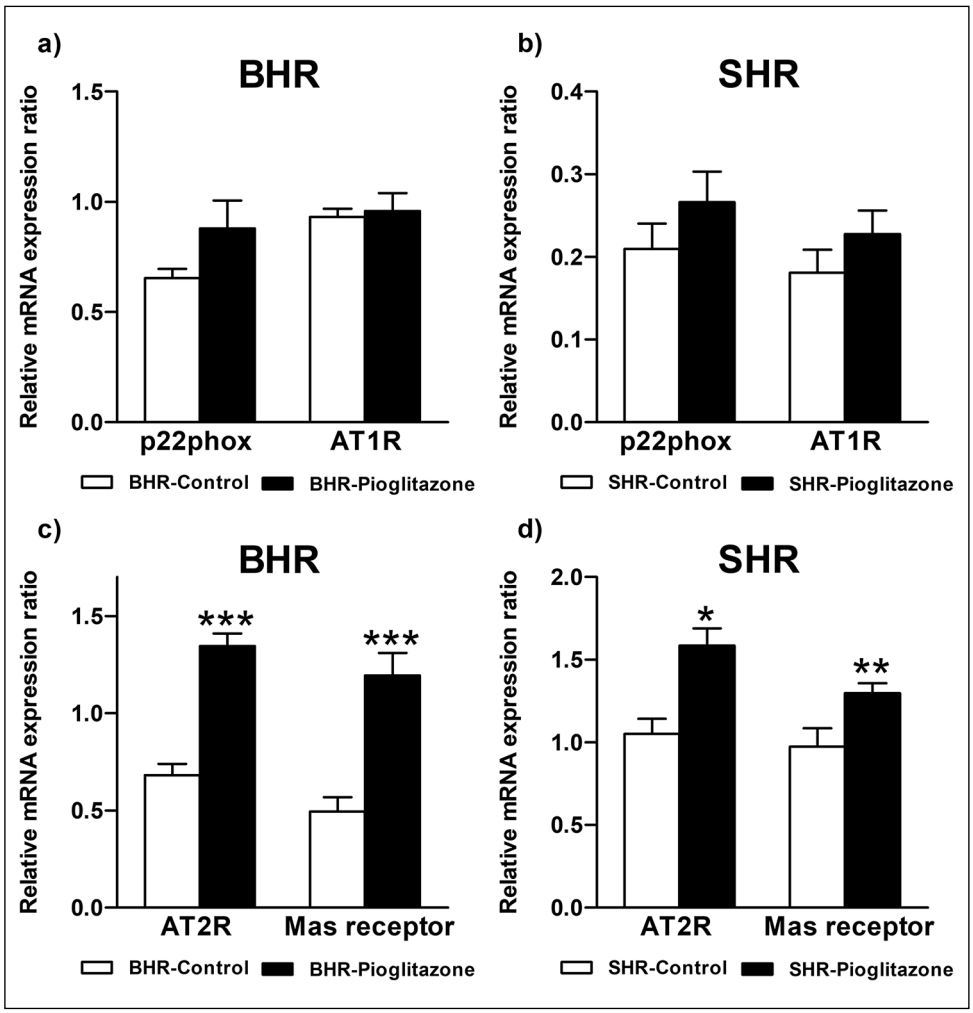

Fig. 5. Effect of pioglitazone on RAS cascade gene expression changes of a) Nox oxidase subunit p22phox and AT1R in BHR; b) Nox subunit p22phox and AT1R in SHR; c) the 'pro-vasodilatatory' AT2R and Mas receptor in BHR and d) the 'pro-vasodilatatory' AT2R and Mas receptor in SHR. Gene expression has been normalized on 'housekeeping' gene β-actin. Control groups (BHR-Control; n = 6 and SHR-Control; n = 6), groups treated with pioglitazone (BHR-Pioglitazone; n = 6 and SHR-Pioglitazone; n = 7). Values represent mean ± SEM; asterisks indicate statistical significance of PIO versus the corresponding Control group (*P < 0.05; **P < 0.01, ***P < 0.001).

subunit, AT1R, AT2R and Mas receptor. The pioglitazone treatment did not significantly influence the mRNA expression of the p22phox Nox subunit (*Fig. 5a*) and the 'pro-vasoconstrictory' AT1R (*Fig. 5b*). PIO up-regulated the 'pro-vasodilatatory' Mas (*Fig. 5c*) and AT2R mRNA expression (*Fig. 5d*) in the renal cortex samples obtained from BHR and SHR.

Effects of PPARγ agonist pioglitazone on Nrf2 and antioxidant system

PPARγ is known to modulate redox homeostasis. We investigated the effects of pioglitazone on antioxidant system activation in the renal cortex. Pioglitazone administration

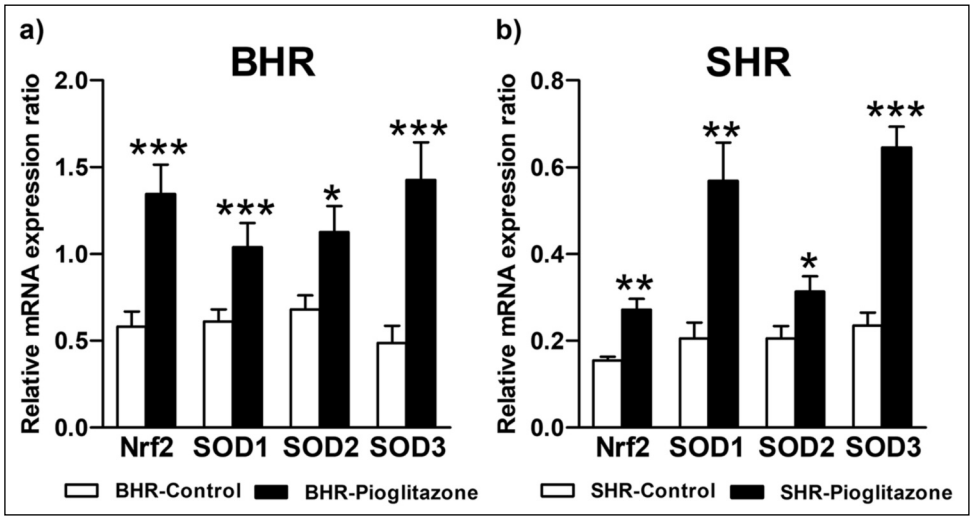

Fig. 6. Effect of pioglitazone on a) gene expression of Nrf2 and SOD isoforms in BHR; b) gene expression of Nrf2 and SOD isoforms in SHR. Gene expression has been normalized on 'housekeeping' gene, β-actin. Control groups (BHR-Control; n = 6 and SHR-Control; n = 6), groups treated with pioglitazone (BHR-Pioglitazone; n = 6 and SHR-Pioglitazone; n = 7). Values represent mean ± SEM; asterisks indicate statistical significance of Pioglitazone versus the corresponding Control group (*P < 0.05; **P < 0.01, ***P < 0.001).

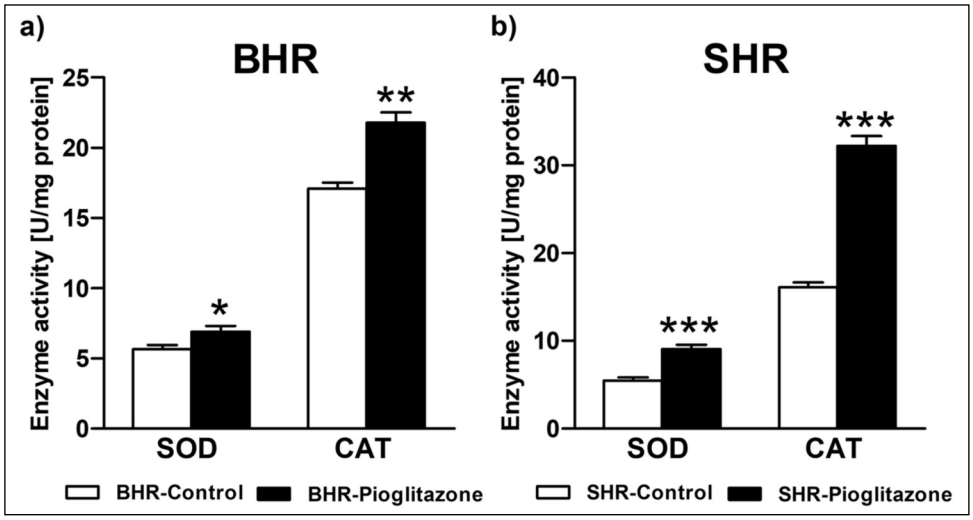

Fig. 7. Effect of pioglitazone on enzyme activities a) total SOD and CAT enzyme activities in BHR, and b) total SOD and CAT enzyme activities in SHR. Control groups (BHR-Control; n = 6 and SHR-Control; n = 6), groups treated with pioglitazone (BHR-Pioglitazone; n = 6 and SHR-Pioglitazone; n = 7). Values represent mean ± SEM; asterisks indicate statistical significance of Pioglitazone versus the corresponding Control group (*P < 0.05; **P < 0.01, ***P < 0.001).

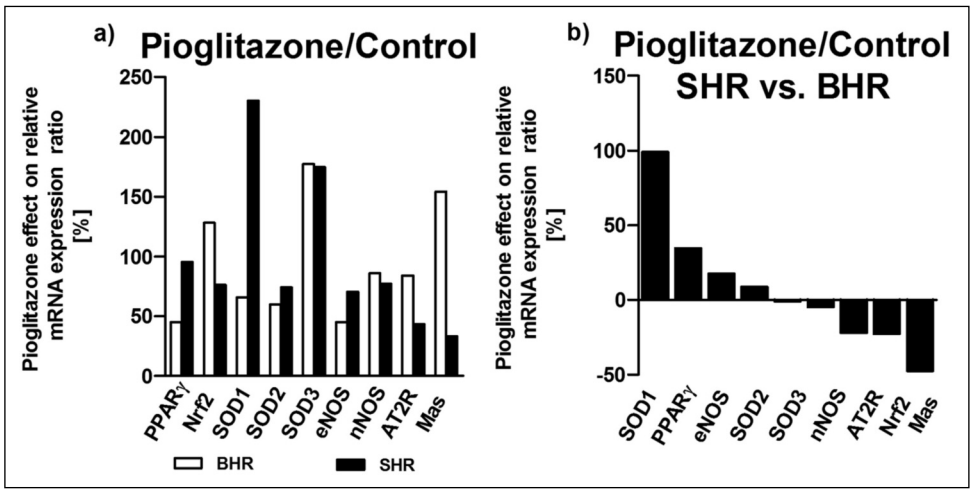

Fig. 8. Differences in pioglitazone effect on gene expressions in SHR and BHR in the kidney. Gene expression has been normalized on 'housekeeping' gene β-actin in all groups.

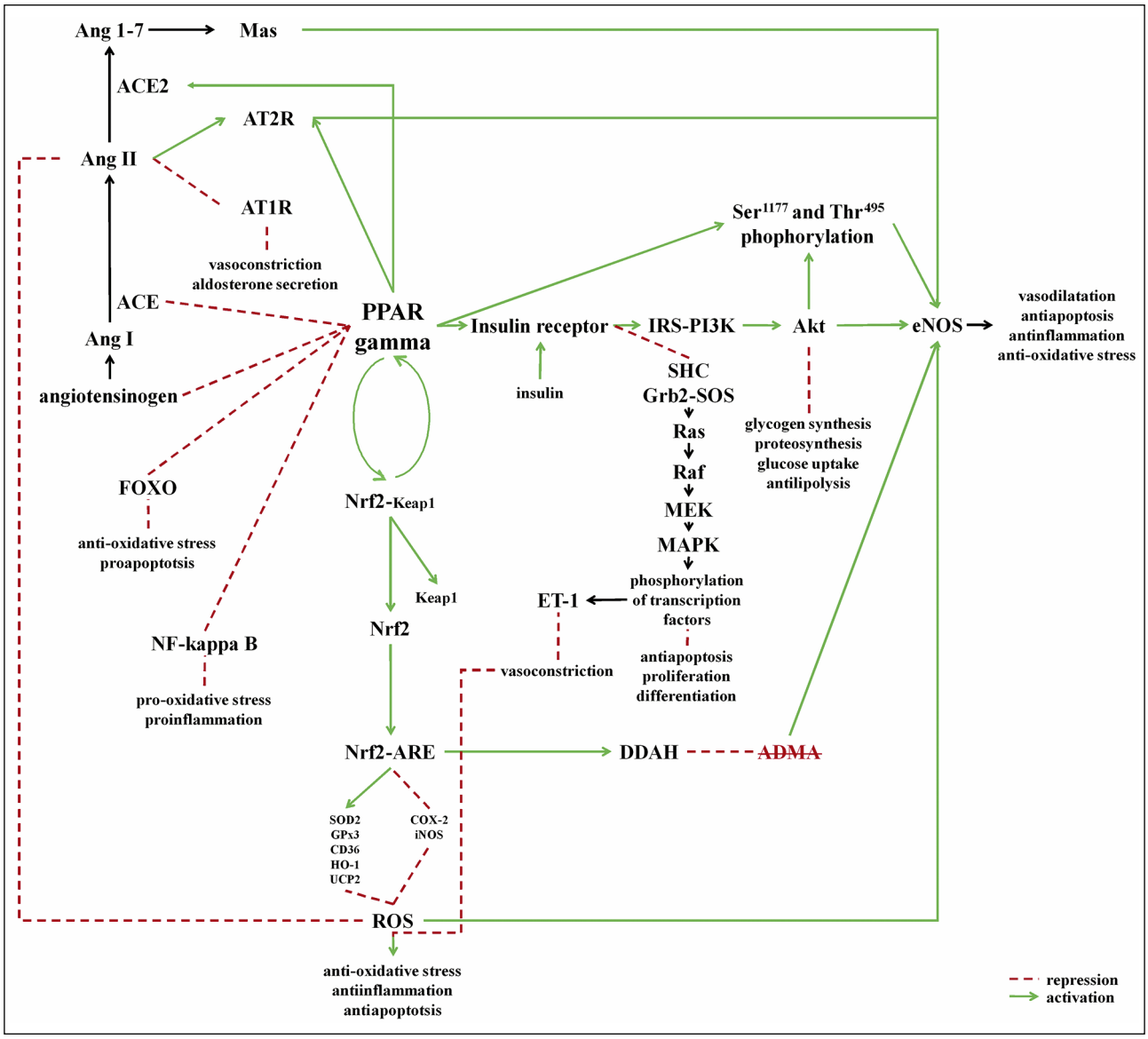

Fig. 9. PPARγ mechanisms of blood pressure regulation.
RAS cascades: left figure part: Ang 1-7; angiotensin 1-7; Ang I, angiotensin I; Ang II, angiotensin II; ACE, angiotensin converting enzyme; ACE2, angiotensin converting enzyme 2; AT1R, angiotensin-receptor 1; AT2R, angiotensin-receptor, Mas, Mas receptor, G protein-coupled receptor.
Nrf2 regulation: central figure part: PPARγ, peroxisome proliferator-activated receptor gamma, ROS, reactive oxygen species; Nrf2, nuclear factor E2-related factor 2; ARE, antioxidant response element; HO-1, heme oxygenase-1; SOD2, superoxide dismutase 2; UCP2, uncoupling protein 2, CD36, cluster determinant 36; COX-2, cyclo-oxygenase 2; iNOS, inducible nitric oxide synthase; Keap1, Kelch-like ECH associated protein 1; GPx3, glutathione peroxidase 3.
PI3K/Akt/NOS pathway: right figure part: IRS-1, insulin receptor substrate 1; PI3K, phosphatidylinositol-3-kinase; Akt, Akt serine/threonine kinase 1; eNOS, endothelial nitric oxide synthase.

influenced the major cell redox sensor, which is nuclear factor Nrf2. Gene expression of Nrf2 was elevated in BHR and SHR treatment groups compared to controls (*Fig. 6a*). We also investigated the activation of Nrf2-dependent antioxidant mechanisms. At mRNA level, we found that pioglitazone treatment increased expression of SOD1, SOD2, and SOD3 compared to control groups (*Fig. 6b*). We also determined the influence of pioglitazone on enzyme activities of SOD and CAT. We found that pioglitazone significantly elevated enzyme SOD activity in the renal cortex of SHR and BHR (*Fig. 7a* and *7b*). Total CAT enzyme activity was also elevated after pioglitazone administration (*Fig. 7a* and *7b*).

Effect of PPARγ activation on differences in expressions of renin-angiotensin system, reactive oxygen species and antioxidant genes between young borderline and spontaneously hypertensive rats

Pioglitazone increased PPARγ activation by 45.0% in BHR and 95.3% in SHR and increased eNOS and nNOS up to 85%. The largest effect of pioglitazone, 150% (BHR) versus 230% (SHR), has been observed in SOD1, SOD3 (177.3% in BHR versus 174.7% in SHR) and the Mas receptor (154.1% in BHR) on both hypertensive groups (*Fig. 8a*). Highest differences in gene expression among SHR versus BHR animals after

pioglitazone treatment (*Fig. 8b*) have been observed in SOD1 (99.1%) and Mas receptor (–47.6%).

DISCUSSION

Experimental and physiological evidence indicate that hypertension development may be affected by the renal PPARγ-dependent mechanisms. Our experimental data showed improvement of BP: administration of pioglitazone decreased BP in BHR and slowed down the development of BP increase in SHR (*Fig. 1*). These observations suggest a PPARγ-dependent regulation of BP through pioglitazone activation in young pre-hypertensive animals.

The decrease of BP can be partially attributed to changes in renal function and sodium excretion. At least three response types have been observed in diabetic patients in response to pioglitazone (38). Several studies report beneficial effects of PPARγ agonists on the kidney (39-42). The study of Rodriguez *et al.* suggests that in animal model of Type 2 diabetes mellitus, treatment with pioglitazone normalized the glomerular filtration (GFR) (43). Nitroso-sulfide coupled signaling triggers can also influenced renal artery outputs. In intrarenal artieries samples of adult patients with a renal cell damage (cell carcinoma, renal pelvic tumor) and arterial hypertension has been observed specificic vasoactive responces - increase and acceleration of vasorelaxant responses. These mechanisms were also shown in SHR animal models (44). Cyclosporin A-induced kidney dysfunction can be improved by using PPARγ agonist. Protective effect of PPARγ is mediated trough reduced expression of profibrotic factor TGF-β, inhibition of inflammatory processes and oxidative stress resulted in inhibition of ROS production and apoptosis (45). All these data suggest that a PPARγ agonist might ameliorate the pre-glomerular arteriole remodelling and renal functions. PPARγ is expressed in several locations of the kidney (1), and modulation of PPARγ activity by TZDs has several direct and indirect effects on sodium balance and blood pressure. The sodium/fluid retention induced by the increase in renal sodium reabsorption is a well-identified side effect of TZDs treatment, observed in up to 5% of diabetic patients treated with TZDs (46).

Creatinine is commonly used as a measure of kidney function for creatinine clearance test and estimation of the GFR - the rate of blood flow through the kidneys. In young BHR and SHR animals we did not observe any significant changes of creatinine in plasma.

Administration of TZD decreases the levels of free fatty acids and lipid storage in adipose tissue, and improves adiponectin level (47, 48). We observed that pioglitazone treatment affected the plasma lipid profile, improved lipid profile of cholesterol, while triglyceride concentrations were not influenced in either BHR or SHR (*Table 1*).

Deregulation of NOS and endothelial integrity contribute to the pathogenesis of several vascular diseases (49). In the kidney, NO has numerous important functions including the regulation of renal hemodynamic, maintenance of medullary perfusion, mediation of pressure-natriuresis, blunting of tubulo-glomerular feedback, inhibition of tubular sodium reabsorption, and modulation of renal sympathetic neural activity (50). Notably, deficient renal NO synthesis has been implicated in the pathogenesis of hypertension. We observed that treatment with pioglitazone increased eNOS and nNOS expression and protein levels as well as NOS activities in both animal treatment groups (*Figs. 3* and *4*). NO-synthases, nNOS, and eNOS play key roles in normal kidney physiology. NO derived from renal nNOS can dilate afferent arterioles and has an important role in the regulation of glomerular blood flow and GFR by regulation of vascular tone of afferent arterioles. Renal nNOS is important for renin secretion, and its expression is regulated by salt (51), renal Ang II and intracellular pH (52). nNOS expression also shows positive correlation with renin production in the granular cells (53). Enhancement of renal nNOS can be a promising strategy to prevent reduction of renal function (54). The nNOS protein expression might be regulated by a PPARγ-dependent mechanism. Wu *et al.* note an increase in nNOS dimer/monomer ratio in the rostral ventrolateral medulla after two weeks of oral intake of pioglitazone, resulting in a decrease of ROS tissue level and restoration of NO bioavailability (4). Pioglitazone has renoprotective effects - it effectively attenuates glomerular hyperfiltration and hyperfiltration-associated glomerular injury in diabetic nephropathy and restores the altered nNOS signaling in macula densa (55).

The renin-angiotensin system is another important player in BP regulation. The main receptors in this system are AT1R, AT2R, and Mas. Recent studies indicate that PPARγ-regulated gene expression can also influence the function of RAS. A specific PPARγ-dependent mechanism that influences development of hypertension is an attenuation of AT1R gene expression (24, 30, 31) and/or inhibition of signaling pathways, such as PI3K and MAPK (30, 56). In our study, pioglitazone administration did not affect mRNA expression of AT1R in young pre-hypertensive animals. Different types of TZDs have been shown to differ in the modulation of AT1R mRNA transcription (57). In the conditions of our study, also age-dependence and hypertension types can change the AT1R mRNA responses. PPARγ can also reduce the levels of Ang II in circulation and tissue, with regulatory effect on vascular NOXs activity (58). In the renal cortex of young pre-hypertensive animals, we have not observed changes in the AT1R/ NOX pathways (*Fig. 5*), while an increase was observed for AT2R and Mas receptor expression (*Fig. 5* and *8*). Another study found similar effects, where PPARγ activation modulates expression of AT2R mRNA (24) and activation of 'alternative RAS axis'. Potentiation of AT2R and Mas receptor expression may result in stimulation of jejunal sodium and water absorption by a pathway that includes stimulation of the SNS and NO production (59, 60). In other experimental studies of nephropathy, treatment of rats with a PPARγ agonist affected renal expression of RAS components, normalizing increased renal expression of ACE and enhancing the expression of Mas receptor (61). The drugs, that inhibit RAAS at different levels such as ACE inhibitors, Ang II antagonists and aldosterone increase a risk of hyperkalemia (62). Plasma potassium levels are markedly increased in model of diabetes mellitus 2. type, which can be significantly decreased by rosiglitazone (63). The major effect of PPARγ agonist pioglitazone observed in our study was on gene expression of the protective axis of the RAS cascade - AT2R and Mas receptor (*Fig. 8*) and PPARγ mechanisms of blood pressure regulation in RAS cascades are involved in *Fig. 9*.

Some studies associate the increased BP in BHR with a higher sympathetic activity (64, 65) and studies using AT2R gene deletion mice suggest the contribution of this receptor to sympathoinhibition (66, 67). While AT1R in the central nervous system has been solidly linked to sympathoexcitation, AT2R activation exhibits opposite influence on sympathetic tone (68).

Modulation of redox homeostasis is closely related to NO bioavailability and RAS cascade and can be affected by PPARγ activation. Our findings indicate that PIO-dependent activation of PPARγ influenced renal aberrant redox regulation through reciprocal transcriptional regulation between Nrf2 and PPARγ. Nrf2 mRNA level was elevated in BHR and SHR after PIO treatment, and these changes were in correlation with a markedly elevated PPARγ mRNA expression (*Fig. 2*).

We investigated activation of Nrf2- and PPARγ-dependent antioxidant mechanisms (*Figs. 6* and *7*). The SOD has PPRE consensus sequence in its promoter region. A promoter analysis of SOD gene revealed a conserved PPRE sequence located in –797 and –792 nt for SOD1 isoform and between –985 and –935 nt in SOD2 (69). Therefore, both are regulated by direct interaction of PPARγ on the PPRE of their promoters under specific conditions. SOD1 (70) and SOD2 (72) isoforms are regulated by Nrf2-dependent action and gene expression of SOD1 and SOD2 after pioglitazone administration can be upregulated *via* PPARγ-dependent activation of SOD1 expression as well as through PPARγ-dependent activation of Nrf2, resulting in SOD1 and SOD2 upregulation. Expression of mRNA SOD3 was also elevated after pioglitazone treatment, but it is currently unclear whether SOD3 expression could be regulated directly through PPARγ activation. We suggest that elevation of SOD3 mRNA expression after pioglitazone administration is upregulated through PPARγ-dependent activation of Nrf2-mediated SOD3 expression and not directly by PPARγ. This is supported by the finding that expression of SOD3 is regulated by the Nrf2-mediated regulation. Similarly to SOD, CAT could be upregulated by PPARγ proper as well as by PPARγ-dependent activation of Nrf2, both resulting in CAT activity increase (71, 72). Girnun *et al.* identified a putative PPRE in the rat catalase promoter located at nucleotides –1027 to –1015 nt with respect to the translation start site (71, 72). PPARγ mechanisms of blood pressure regulation in Nrf2 regulation are involved in *Fig. 9*.

Our data suggest that renal PPARγ-dependent mechanisms may affect BP regulation in both young pre-hypertensive animal strains. Experimental data show that the etiology of hypertension in SHR involves systemic and renal oxidative stress (74). Renal oxidative stress is thought to play an integral role in progressing hypertension. Interestingly, our data indicate that in SHR pioglitazone affects BP regulation mainly through oxidative stress reduction by elevated SOD1 expression. Although oxidative stress is present in both SHR and BHR, oxidative imbalance is more pronounced in SHR (75, 76). In SHR, oxidative stress appears to be the cause of hypertension development on a larger scale and the major effect of PPARγ activation is the reduction of oxidative stress.

We focused and observed more pronounced increase in PPARγ expression in SHR than in BHR, probably a compensatory response. In another study, authors showed that pioglitazone treatment of Albino Wistar rats has no significant effect on concentrations and activities of liver and kidney function markers in pioglitazone treated groups compared to normal controls (77). Diep *et al.* suggest that increased PPARγ expression may play a compensatory role in remodelling of blood vessels in reaction to hypertensive vascular growth in SHR (78). In BHR, the major effect of PIO was on changes in the protective axis of the RAS cascade (AT2R and Mas receptor). Increased BP is associated with a higher sympathetic activity in BHR (64, 65). Our data suggest that pioglitazone-dependent activation of PPARγ in BHR might influence regulation of BP and hypertension development through the already mentioned pro-vasodilatatory RAS axis.

Associations between cause and effect to renal function and blood pressure control has been observed in Tain *et al.* renal studies where was detected transcriptome analysis of programmed hypertension (79). These data have been studied in 4 models (pregnant Sprague-Dawley rats with coloric restriction (CR), streptozotocin models, high-fructose (HF) diet, or high salt model). All four models induced programmed hypertension and 16 shared genes in a two-week-old kidney among four different models. The peroxisome proliferator-activated receptor (PPAR) signaling pathway and glutathione metabolism pathway were shared by the CR, diabetes, and HF models. Experiments to determine the contribution of these pathways to renal function and blood pressure control in SHR and BHR treated with pioglitazone were finalised in our data outputs. Signaling pathways through gene responces in kidney in young hypertensive model animals and all system outputs and interactions are displayed in *Figs. 8* and *9*.

Conclusion

Blood pressure regulation is an important and complex coaction of various factors, where a normal kidney function plays a considerable role. In the kidney, NO has numerous important functions, in particular, its role in phosphorylation of NOS isoforms at both activating and inhibitory sites is emerging in the regulation of NO synthesis in the kidney. The largest effect of PPARγ has been observed in SOD1, SOD3 and Mas receptor genes. Differences between BHR and SHR were found in the effects of PPARγ on SOD1 and Mas receptor expressions, where SHR expression was opposite to that observed in BHR.

The improvements of NO availability, AT2R and Mas receptors, and aberrant redox regulation are thought to be the major correlated mechanisms mediating the BP decrease observed in treatment with the PPARγ agonist.

Abbreviations: 15d-PGJ2, 15-deoxy-Δ12,14-PGJ2; ACE, angiotensin converting enzyme; ACE2, angiotensin converting enzyme 2; ADMA, asymmetric ADMA; Akt, Akt serine/threonine kinase 1; Ang 1–7, angiotensin 1–7; Ang I, angiotensin I; Ang II, angiotensin II; ARE, antioxidant response element; AT1R, angiotensin receptor 1; AT2R, angiotensin-receptor 2; BHR, borderline hypertensive rats; BP, blood pressure; BW, body weight; CAT, catalase; CD36, cluster determinant 36; cDNA, complementary DNA; CNS, central nervous system; COX-2, cyclo-oxygenase 2; DDAH, dimethylarginine dimethylaminohydrolase; DR1, direct repeat spaced by one nt; eNOS, endothelial nitric oxide synthase (NOS3); ERKs, extracellular signal regulated kinases; ET-1, endothelin-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; glc, glycemia; GPx3, glutathione peroxidase 3; H_2O_2, hydrogen peroxide; HDL, plasma-high density lipoproteins concentration; HO-1, heme oxygenase-1; HUVECs: human umbilical vein endothelial cells; HW/BW, heart weight/body weight; iNOS, inducible nitric oxide synthase (NOS2); IRS-1, insulin receptor substrate 1; Keap1, Kelch-like ECH associated protein 1; LDL, plasma-low density lipoproteins concentration; LV, left heart ventricle; LVW/BW, left ventricle weight/body weight; MAPK, mitogen-activated protein kinase; Mas, Mas receptor, GPCRs, G protein-coupled receptor; mRNA, messenger RNA; nNOS, neuronal nitric oxide synthase (NOS1); NO, nitric oxide; NOS, nitric oxide synthase; Nox, NADPH oxidase; Nrf2, nuclear factor E2-related factor 2; $ONOO^-$, peroxynitrite; p22phox, subunit of Nox; PI3K, phosphatidylinositol-3-kinase; PIO, pioglitazone; PPARγ, peroxisome proliferator-activated receptor gamma; PPRE, PPAR response element; qRT-PCR, real-time quantitative polymerase chain reaction; RAS, renin-angiotensin system; ROS, reactive oxygen species; RSG, rosiglitazone; RXR, retinoid X receptor; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SHR, spontaneously hypertensive rats; siRNA, small interfering RNA; SNS, sympathetic nervous system; SOD, superoxide dismutase; SOD1, superoxide dismutase 1 (Cu/ZnSOD); SOD2, superoxide dismutase 2 (MnSOD); SOD3, superoxide dismutase 3, extracellular SOD (ecSOD); TAG, plasma triglyceride concentration; TZDs, thiazolidinedione; UCP2, uncoupling

protein 2; VSMCs: vascular smooth muscle cells; WST-1, water-soluble tetrazolium salts 1.

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