

Age-dependent effect of PPAR γ agonist pioglitazone on kidney signaling in borderline hypertensive rats

Linda Grešová¹, Miroslava Kvandová^{1,2}, Peter Kvasnička³ and Ima Dovinová¹

¹ *Institute of Normal and Pathological Physiology, Centre of Experimental Medicine, Slovak Academy of Sciences, Bratislava, Slovakia*

² *Center for Cardiology, Cardiology I - Laboratory of Molecular Cardiology, University Medical Center of the Johannes Gutenberg-University Mainz, Mainz, Germany*

³ *Institute of Particle and Nuclear Physics, Faculty of Mathematics and Physics, Charles University, Prague, Czech Republic*

Abstract. The peroxisome proliferator-activated receptor γ (PPAR γ) is a nuclear receptor and nutrition factor which takes part in the cellular signaling by several agonists such as pioglitazone. PPAR γ can serve as potential target in treatments of metabolic syndrome diseases and/or hypertension. In the present study we investigated the effects of pioglitazone, a PPAR γ agonist, on hypertension development in young and adult borderline hypertensive rats (BHR). In renal signaling we observed connections between PPAR γ and Nrf2, antioxidant in adult animals and differences between young and adult BHR in Nrf2-activated detoxificant outputs (NQO1, HO-1) and NO-synthases. Blood pressure in animals had been detected by cuff plethysmography, cell signaling in the kidney was studied by gene expression determination using qPCR, and nitric oxide synthase (NOS) activity was measured by radioactive detection. Pioglitazone treatment in adult BHR caused no detectable changes in antioxidant and detoxificant responses. The main effects were observed in blood pressure improvement, endothelial NOS expression and NOS activities in both young and adult BHR.

Key words: Age-dependence — BHR — Kidney — PPAR γ — Nrf2 — NOS — Hypertension

Introduction

The peroxisome proliferator-activated receptor γ (PPAR γ) is a nuclear receptor and nutrition factor in hypertension (Kvandová et al. 2016; Tain et al. 2016). The activity of PPAR γ is influenced by a variety of extracellular ligands - glitazones (rosiglitazone, pioglitazone and troglitazone) and intracellular ligands - prostaglandins, leukotrienes, and α -lipoic acid (Houseknecht et al. 2002).

PPAR γ activation takes part in the regulation of lipid metabolism, glucose homeostasis and anti-inflammatory response as well as in several cell signaling pathways (Polvani et al. 2012). PPARs undergo a conformational change that causes the translocation to the nucleus and the heterodimerization with another nuclear receptor, the retinoid X receptor

(RXR) (Mello et al. 2009). The PPAR-RXR heterodimer then binds a DNA portion in the promoter region of target genes, called peroxisome proliferator response element (PPRE), modulating the expression of several genes involved in different physiological or pathological processes (Kliwer et al. 1992). PPAR γ and Nrf2-ARE system are connected by a positive feedback. PPAR γ may also regulate and modulate other systems, such as the renin-angiotensin system (RAS) and NO-synthases (Kvandová et al. 2016, 2017). PPAR γ activation may improve dysregulation in metabolic syndrome and hypertension (Chan et al. 2010; Dovinová et al. 2013).

Blood pressure (BP) regulation is an important and complex process. There are several mechanisms that coordinate BP outputs. Nrf2, which is a redox-sensitive nuclear transcription factor exposed to oxidative stress or noxious attacks. It is involved in the induction of antioxidative, detoxificant, and other cytoprotective genes, which provide protection against oxidative stress-induced damage in a variety of cardiovascular diseases and in hypertension (Majzunova et al. 2013; Barančík et al. 2016). The positive

Correspondence to: Ima Dovinová, Institute of Normal and Pathological Physiology, Centre of Experimental Medicine, Slovak Academy of Sciences, Sienkiewiczova 1, 813 71 Bratislava, Slovakia
E-mail: ima.dovinova@savba.sk

feedback loop between PPAR γ and Nrf2 sustains the expression of both transcription factors and their antioxidant genes. Activation of Nrf2 and PPAR γ pathways has ameliorating effects on metabolic disorders and injuries caused by oxidative stress (Lee 2017; Kvandova et al. 2018). After activation, Nrf2 enters nucleus, binds to the anti-oxidant response element (ARE) and produces NAD(P)H:quinone oxidoreductase-1 (NQO1), heme oxygenase-1 (HO-1), glutathione S-transferase, as well internal antioxidants of different superoxide dismutase (SOD) isoforms (Li et al. 2008; Lob et al. 2010).

In BP regulation and generally in the cardiovascular system, NO bioavailability plays a protective role in several tissues (vascular tissues, brain stem, kidney). Oral intake of pioglitazone (PPAR γ agonist) significantly abrogated adverse molecular events in metabolic syndrome rats and increased NO level in rostral-ventrolateral medulla of brain stem (Wu et al. 2014). PPAR γ can modulate several signaling pathways: it affects the insulin signaling pathway by modulation of expression and/or phosphorylation of signaling molecules through the PI3/Akt/eNOS pathways (Kvandová et al. 2017). Deficiency of endothelial NO-synthase (eNOS) due to endothelial cell dysfunction plays an important role in the pathophysiology of cardiovascular diseases (hypertension, atherosclerosis) as well as in renal injuries (Nakagawa et al. 2011). Aging is a universal process that affects all organs. Age-related disruptions in cellular homeostasis result in decline of organ functions and responsiveness to physiological stress. A gradual decline in renal function occurs in most healthy individuals as they age (Coresh et al. 2003), and the amount of glomerular, vascular and interstitial scarring in the renal tissue of healthy adults increases with age (Rule et al. 2010).

In the present study we investigated the effects of pioglitazone in the hypertension model of young and adult borderline hypertensive rats (BHR). Our study was focused on changes in BP and on renal signaling in young and adult BHR animals after treatment with PPAR γ agonist pioglitazone. We studied the effects of pioglitazone on BP development and on the components of the antioxidant SOD1-3 and detoxificant NQO1 and HO-1 responses, and on the NOS signaling pathway.

Materials and Methods

Experimental design – animal model and treatment protocol

In our study young (5-week old) and adult (12-week old) male borderline hypertensive rats (BHR; offspring of spontaneously hypertensive dams and Wistar-Kyoto sires) were used. The rats were allowed one week to acclimatize to the lab conditions, and were housed three *per* cage: temperature 22–24°C, humidity 45–60%, a 12/12 h light/

dark cycle. Young and adult BHR animals were divided into two groups: control group (treated with saline solution) and PIO group (treated with pioglitazone by oral gavage, 10 mg/kg/day during 10 days). All animal experiments were approved by the Department of Animal Wellness, State Veterinary and Food Administration of the Slovak Republic and carried out 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 a Statham Pressure Transducer P23XL (Hugo Sachs, Germany). All groups of rats (control group and PIO group of young and adult BHR) have been measured between 08:00 a.m. and 11:00 a.m. as described in detail previously (Puzserova et al. 2013). BP measurements were performed before, during (every three days), and after the treatment period. Each value was calculated as mean of seven measurements.

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 TRIsure reagent (Bioline) according to manufacturer's protocol. The isolated total RNA was quantified spectrophotometrically at 260 nm using a Nanodrop 2000 UV-VIS (Thermo Scientific, USA). The purity of RNA was measured at 260/280 nm (rate ~2.0) and 260/230 (rate range: 1.8–2.2) for elimination of protein, phenol or other contaminants.

cDNA preparation

Reverse transcription reaction was performed using a SensiFAST™ cDNA Synthesis Kit (Bioline, UK) on the Mastercycler Personal (Eppendorf, Germany) according to manufacturer's protocol. Real-time polymerase chain reaction by amplification of cDNA was performed on a CFX96 Real-Time PCR detection system (Bio-Rad, USA) using the Sensi FAST SYBR No ROX kit (Bioline, UK). PCR reaction for each sample was carried out in duplicate for all cDNA. Samples were measured using the Bio-Rad CFX Manager Software (version 2.0). Specific primer pairs were used to amplify the genes studied (PPAR γ , Nrf2, HO-1, NQO1, SOD1, SOD2, SOD3 and eNOS). β -actin was used as the “housekeeping” gene (Dovinová et al. 2013, Kvandova et al. 2018). All chemicals used in this study were purchased from Sigma-Aldrich (Germany), Merck Chemicals (Germany); primers were from Metabion GmbH (Germany).

Determination of nitric oxide synthase (NOS) activity

Total enzyme activity of NOS was determined in 20% tissue homogenates of the renal cortex using measurement of the conversion of radioactive [^3H]-L-arginine (MP Biomedicals, USA) to [^3H]-L-citruline (Bernatova et al. 2002). The product was detected by a Tri-Carb 2910TR liquid scintillator (PerkinElmer, USA) and results were expressed in pmol/min/mg of tissue proteins, determined by the Lowry method.

Statistical analysis

Data are presented as mean values \pm standard deviation (SD) and the number (n) of independent measurements. BP was analyzed by two-way ANOVA.

Statistical analysis of gene expressions was analysed by one-way ANOVA and unpaired Student's t -test and was carried out using an R language script (R Development Core Team, 2008). We carried out two analyses, one for the effect of pioglitazone on gene expression in adult BHR, the other on the effect of pioglitazone on expression of selected genes in adult and young BHR. A mixed-factor ANOVA model was fitted to each of the two data sets using the R's nlme package (Pinheiro et al. 2018), with contribution of individual animals as random factor. For *post-hoc* comparisons, we used the glht method of the multcomp R package (Hothorn et al. 2008), with single-step FWER-controlling significance adjustment. Differences were marked as significant for * $p < 0.05$ in all tests.

Results

Effect of the pioglitazone on blood pressure in BHR

The effects of treatment with the PPAR γ agonist pioglitazone significantly (* $p < 0.05$) decreased the final BP. Changes of final BP between the control and PIO groups were 121.5 mmHg vs. 115 mmHg in young BHR and 148.5 mmHg vs. 136.5 mmHg in adult BHR. The interim BP measurements document a stable BP in young BHR and slightly increasing BP in adult BHR control groups. In the PIO groups, BP monotonically and uniformly decreased during the treatment period in both young and adult rats.

Effect of the pioglitazone on Nrf2 outputs in the kidney of BHR

Nrf2 target genes, HO-1 and phase II-detoxifying gene NQO1, were detected in tested BHR after pioglitazone treatment. PPAR γ may act directly for Nrf2 activation in kidney, which may be the underlying mechanism of its renoprotective effect (Li et al. 2008). Figure 1 shows that in young

BHR both Nrf2 signaling outputs in the kidney, HO-1 and NQO1 gene expression, were significantly increased, while no such response was observed in adult BHR.

Pioglitazone treatment and administration markedly elevated PPAR γ mRNA and Nrf2 mRNA levels and increased gene expression of SOD1, SOD2, and SOD3 compare to control group in young BHR (Kvandová et al. 2018). Table 1 lists PPAR γ , Nrf2, and SOD isoforms gene expressions, which were not significantly changed in adult BHR.

Effect of the pioglitazone on NO-synthases in the kidney of BHR

In the kidney of young BHR, we observed a significant increase of mRNA eNOS as well as NOS activities. The same effect, with yet higher outputs, was observed in the kidney of adult BHR (see Figure 2).

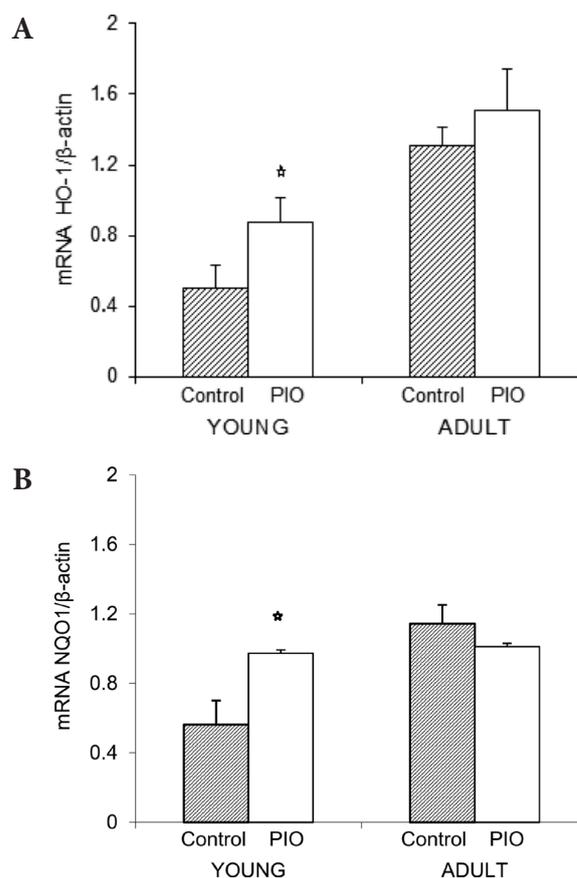


Figure 1. Effect of pioglitazone on gene expression of HO-1 (A) and NQO1 (B) in the kidney of young and adult BHR. Gene expression was normalized on the “housekeeping” gene, β -actin. Values represent mean \pm SD, * $p < 0.05$ versus control group. BHR, borderline hypertensive rats; PIO, rats treated with PPAR γ agonist pioglitazone; $n = 6$ (for young Control, young PIO group, and adult PIO group), $n = 5$ (for adult Control).

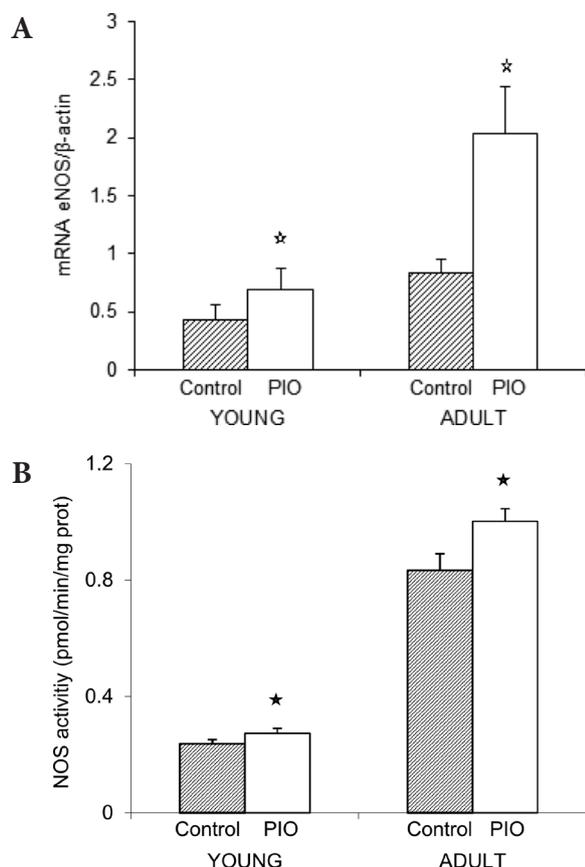


Figure 2. Effect of pioglitazone on gene expression of eNOS (A) and NOS activities (B) in the kidney of young and adult BHR. Gene expression was normalized on the 'housekeeping' gene, β -actin. Values represent mean \pm SD, * $p < 0.05$ versus control group. BHR, borderline hypertensive rats; PIO, rats treated with PPAR γ agonist pioglitazone; $n = 6$ (for young Control, young PIO group, and adult PIO group), $n = 5$ (for adult Control).

Discussion

Hypertension is an important facet of the metabolic syndrome. Adulthood hypertension and metabolic syndrome can be programmed in response to nutritional insults in early life. PPARs serve as nutrient-sensing signalling, linking nutritional programming to hypertension and the metabolic

syndrome. PPAR γ are expressed in the kidney and are involved in BP control (Tain et al. 2016). Some studies suggest that administration of a PPAR γ agonist (e.g. rosiglitazone) to spontaneously hypertensive rats (SHR) may achieve a BP reduction effect by different mechanisms in different stages of hypertension (Li et al. 2010).

In the highly hypertensive rats, SHR, the PPAR γ agonist pioglitazone decreases BP only in young animals (Dovinová et al. 2013), but not in adult SHR (Kvandova et al. 2015). In the present study of borderline hypertension in BHR, pioglitazone treatment improved BP. Significant BP decrease was observed in both young and adult BHR.

BP regulation is an important and complex process including a variety of factors, where normal kidney function plays a considerable role. Experimental and physiological evidence indicates that hypertension development may be affected by the renal PPAR γ -dependent mechanisms (Tain et al. 2016). In our experiments, we observed no increase in PPAR γ and Nrf2 expression in the kidney of adult BHR (Table 1), while pioglitazone administration markedly elevated PPAR γ and Nrf2 mRNA levels in young BHR as has been observed previously in young BHR in a similar study (Kvandova et al. 2018). PPAR γ -dependent activation resulting in SOD1 and SOD2 upregulation and PPAR γ -dependent activation of Nrf2-mediated elevation of SOD3 expression in young BHR has also been reported by other authors (Zhu et al. 2005; Kvandova et al. 2018). Similar differences between young and adult BHR were also observed in Nrf2 outputs, HO-1 and NQO1 (Figure 1).

In the cardiovascular system, endothelial nitric oxide synthase (eNOS) contributes to the regulation of blood flow and BP and in experimental animals deficiency of eNOS $^{-/-}$ produces hypertension with lower circulating nitrite levels (Wood et al. 2013). eNOS is an essential enzyme to the maintenance of cardiovascular integrity by producing NO, which is a key molecule with multiple functions, including vasodilation. Therefore, dysregulation of eNOS is thought to contribute to pathogenesis of certain vascular diseases, such as atherosclerosis and hypertension. eNOS is regulated not only at the level of expression, but also non-genomically, by subcellular targeting, protein-protein interactions, fatty acylation, and phosphorylation. Several specific phosphorylation sites responsible for eNOS activation have been identified. In contrast, there is evidence of phosphorylation

Table 1. Effect of pioglitazone on gene expression of PPAR γ , Nrf2, and SOD isoforms in adult BHR

Group	PPAR γ	Nrf2	SOD1	SOD2	SOD3
Control ($n = 5$)	1.74 \pm 0.654	1.49 \pm 0.391	1.16 \pm 0.285	2.30 \pm 0.659	1.23 \pm 0.523
PIO ($n = 6$)	1.46 \pm 0.401	1.21 \pm 0.404	1.44 \pm 0.393	1.82 \pm 0.659	1.75 \pm 0.604

Gene expression was normalized on the "housekeeping" gene, β -actin. Values represent mean \pm SD. BHR, borderline hypertensive rats; PIO, rats treated with PPAR γ agonist pioglitazone.

sites that decrease eNOS activity. It has been shown that treatment with troglitazone increases NO production by a PPAR γ -dependent pathway and a PPAR γ -independent direct effect on eNOS phosphorylation (Cho et al. 2004). Thiazolidinediones (TZD) – PPAR γ agonists – are also known to act in part through AMP-activated protein kinase (AMPK) activation. There is growing evidence that AMPK plays a decisive role in normal renal physiology and pathogenesis of hypertension. (Tain and Hsu 2018). In the vasculature, activation of endothelial AMPK has been shown to phosphorylate eNOS¹¹⁷⁷, stimulating NOS activities by NO release and subsequent vasodilatation of both large conduit and resistance arteries (Fu et al. 2016). In our experiments on adult BHR we did not observed changes in PPAR γ expression, but eNOS expression was increased (Figure 2A). eNOS expression can be activated through PPAR γ -independent direct effect. It seems that the effect in adult rats was achieved through pioglitazone action. Pioglitazone administration in young and adult BHR improves NOS activities (Figure 2B). Thus, further examination is required to understand the protective effects of TZDs in programmed hypertension and kidney disease, which are exerted *via* PPAR γ -independent direct effect or *via* the PPAR γ signaling pathway.

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