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RESEARCH

# Regulation of tight junction proteins and cell death by peroxisome proliferator-activated receptor γ agonist in brainstem of hypertensive rats

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## Abstract

The decrease in tight junction proteins and their adapter proteins in the hypertensive brain is remarkable. Here, we aimed to investigate tight junction proteins and peroxisome proliferator-activated receptor (PPARγ) activation as well as inflammation factors and cell death proteins in the brainstem of hypertension models, namely spontaneously hypertensive rats (SHR) and borderline hypertensive rats (BHR). At first, SHR and BHR groups were treated with PPARγ agonist, pioglitazone. Then, occludin, claudin-1, claudin-2, claudin-12, ZO-1, and NF-κB p65 gene expression levels; pIKKβ, NF-κB p65, TNF, IL-1β, caspase-3, caspase-9 levels, and PARP-1 cleavage were evaluated. Significantly lower pIKKβ, NF-κB p65, TNF, and IL-1β levels were measured in pioglitazone-treated SHR. Results from this study confirm higher occludin (1.35-fold), claudin-2 (7.45-fold), claudin-12 (1.12-fold), and NF-κB p65 subunit (4.76-fold) expressions in the BHR group when compared to the SHR group. Pioglitazone was found effective in terms of regulating gene expression in SHR. Pioglitazone significantly increased occludin (8.17-fold), claudin-2 (2.41-fold), and claudin-12 (1.85-fold) mRNA levels, which were accompanied by decreased cleaved caspase-3, caspase-9 levels, PARP-1 activation, and proinflammatory factor levels in SHR ($p < 0.05$). Our work has led us to conclude that alterations in tight junction proteins, particularly occludin, and cell death parameters in the brainstem following PPARγ activation may contribute to neuroprotection in essential hypertension.

**Keywords** Cell death · Hypertension · Pioglitazone · Tight junctions

## Introduction

The brain regions known as cardiovascular centers (the nucleus of the solitary tract, caudal ventrolateral medulla, and rostral ventrolateral medulla) are control points of the sympathetic pathway and play critical roles in homeostatic regulation of blood pressure in the short and long term (Esler 2010; Lohmeier and Iliescu 2015). High blood pressure can cause serious complications by affecting organs and vessels, such as kidney failure, stroke, heart damage, and vascular endothelial dysfunction (Fisher and Fadel 2010). Uncontrolled passage of molecules caused by disrupted blood–brain barrier is reported in spontaneous hypertension.

Tight junction proteins (TJPs) form a diffusion barrier by controlling the selective permeability of the membrane for compounds. Also, they limit the apical-basolateral diffusion of membrane components that maintain cell surface polarity. Claudin family members and occludin are the major constituents of this complex (Poliak et al. 2002; Günzel and Yu

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2013). These proteins, and their adapter proteins which bind endothelial cells to the cytoskeleton, are frequently affected by pro-inflammatory cytokines in acute and chronic diseases of the brain (Daniels et al. 2014; Erdo et al. 2017). The loss of their function leads to blood–brain barrier breakdown and more destructive consequences, such as neuronal damage.

In addition to basic functions, TJPs proteins are associated with signal transduction pathways (Matter and Balda 2003; Tan et al. 2004). Apoptosis regulated by PI3K/Akt and mitogen-activated protein kinase (MAPK) signaling pathways is influenced by the members of tight junctions in cancer cells and normal cells in a different manner (Hoevel et al. 2004; Wang et al. 2018; Kuo et al. 2019). In the absence of occludin, apoptosis is triggered by the downregulation of Akt signaling in liver cells (Murata et al. 2005). Thus, the regulation of TJP expressions, particularly occludin, with pharmacological agents may assist neuroprotection via regulating cellular responses associated with apoptosis.

The control of apoptosis and inflammation in the brain is accepted as the most relevant cellular response pathways for neuronal maintenance. It is well documented that proinflammatory cytokines contribute to hypertension.

A transcription factor, nuclear factor-kappa B (NF-κB), is a key regulator of inflammation and pivotal for the regulation of apoptosis (Hoffmann and Baltimore 2006). It is involved in the production of proinflammatory cytokines such as TNF-α and interleukins, as well as the induction of oxidative stress. By phosphorylating NF-κB inhibitors, an inhibitor of nuclear factor kappa-B kinase subunit (IKK-β) activates NF-κB. Following IKK-β-mediated inhibitor modification, NF-B is translocated into the nucleus and activates transcription of several genes involved in immune response, growth control, or apoptosis.

It is a known fact that early diagnosis and treatment are required for hypertension. Although mortality rates have decreased significantly with the discovery of antihypertensive drugs, more than 30% of people remain hypertensive (Calhoun et al. 2008). Peroxisome proliferator-activated receptors (PPARs) are ligand-dependent transcription factors and are therapeutic targets due to their potential regulatory effects in hypertension (Kvandova et al. 2018; Grešová et al. 2019). These receptors are widely expressed in the heart, vascular system, kidney, muscles, and other tissues. They are involved in the regulation of lipid and carbohydrate metabolism, smooth muscle proliferation, migration, and apoptosis, inflammation, atherosclerosis, and other pathological processes (Delerive et al. 2001; Szkup et al. 2018). Up to now, no systematic study has yet been conducted in the brainstem to show how PPARγ activation affects mRNA expression of TJPs and apoptosis during hypertension in the brainstem.

The current study sought to investigate the relationship between tight junction proteins and PPARγ activation, as well as neuroinflammation and cell death in hypertension brainstems. We focused on occludin, claudin-1, claudin-2, claudin-12, and zona occludens-1 (ZO-1) because of their distinct roles in tight junctions and their possible connection with cell death.

At first, we aimed to evaluate possible changes in gene expression levels of selected TJPs in the brainstem of animal hypertension models following PPARγ agonist, pioglitazone (PIO) treatment. Secondly, the changes in the levels of proinflammatory factors (TNF, IL-1β, p-IKKβ, NF-κB p65), the activation of caspase-9 and caspase-3, and cleavage of poly (ADP-ribose) polymerase (PARP) were determined to examine the effects of PIO on neuroinflammation and neuronal death in the brainstem.

## Materials and methods

### Experimental design

In this study, adult (12-week-old) male Wistar-Kyoto rats (control group), 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 1 week to acclimate to the lab conditions, housed three per cage at constant temperatures 22–24 °C and humidity (45–60%) with a 12/12 h light/dark cycle (lights on at 6 a.m. and off at 6 p.m.) and fed a standard pellet diet with tap water ad libitum.

All animal experiments were approved by the Department of Animal Wellness, State Veterinary and Food Administration of the Slovak Republic (EÜHADYEK, 2019–071) 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.

The BHR and SHR animals were divided into two groups: a group treated with saline solution ($n = 6$), and a group treated with pioglitazone ($n = 6$). Pioglitazone (E6910, Sigma-Aldrich, St. Louis, MO, USA) in saline solution was administered by oral gavage, at a dose of 10 mg/kg/day for 10 days (Dovinova et al. 2013). The daily food and water intake and blood pressure (BP) were measured before, during (in 3-day intervals), and after the treatment period.

At the end of the experiment, the animals were sacrificed, brainstems were rapidly dissected. The samples were frozen in liquid nitrogen and stored at − 80 °C until further use. The process of randomization involved first assigning and labeling each animal with a three-digit number and then randomly allocating nonsequential numbers into various treatment groups. After the first medication administration, the allocation key was concealed, and experimenters were

blinded for the rest of the trial. In order to obtain statistically significant results, sample size calculations were performed via power analysis from previous data sets to determine the minimum required number of animals in the study.

## Blood pressure measurement

Systolic BP was measured with a Statham Pressure Transducer P23XL (Hugo Sachs, Germany) and a tail cuff plethysmography, as was already described (Grešová et al. 2019). It is important to note that all blood pressure measurements were consistently taken during the morning hours (between 8:00 and 11:00 am) across all subjects, to account for potential variability due to circadian rhythms. This standardized timing for BP measurements ensures that the data gathered are consistent and comparable across the entire study population.

## Biochemical analysis

### Determination of TNF and IL-1β levels

The concentration of proinflammatory factors was determined using commercially available enzyme-linked immunosorbent assay (ELISA) kits following the instructions (201–11-0765 for TNF, 201–11-0120 for IL-1β, both from SunRed Biological Technology, Co., Ltd. Shanghai). The results are presented as ng of cytokine per mg protein for TNF and pg of cytokine per mg protein for IL-1β.

### Gene expression analysis

Total RNA was extracted using the MasterPure RNA Purification Kit (MC85200, Epicentre Biotechnologies, Madison, WI, USA). The concentration of RNA in each sample was measured at 260 nm, and its integrity was assessed after electrophoresis (data not shown). Prior to the reverse transcription reaction, potentially contaminating residual genomic DNA was eliminated with DNAse I (EN0521, MBI Fermentas, Burlington, Canada). 1 µg/µL total RNA was used for first-strand complementary DNA (cDNA) synthesis by MMuLV reverse transcriptase (28,025,021, MBI Fermentas, Burlington, Canada).

Occludin (GenBank ID: NM_031329.2), Claudin 1 (GenBank ID: NM_031699.2), Claudin 2 (GenBank ID: NM_001106846.2), Claudin 12 (GenBank ID: NM_001100813.1), ZO-1 (GenBank ID: NM_001106266.1), NF-κB p65 (Rela) (GenBank ID: NM_199267.2), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (GenBank ID: NM_017008.4) primers were newly designed using Primer3 software (Untergasser et al. 2012). The forward and reverse primers are shown in Table 2.

Conditions for PCRs were optimized in a gradient cycler with regard to primers and various annealing temperatures. Amplified cDNA samples were assessed by Real-time PCR (LightCycler® 480 System, Roche). Both cDNA synthesis and PCR amplifications included negative control reactions, which were set up by excluding RNA and DNA templates, respectively. The amplification specificity of the PCR products was confirmed by the melting curve analysis (data not shown). GAPDH gene was used as an endogenous control for normalization. The relative expressions of target genes were quantified according to ABI Prism 7700 Sequence Detection System User Bulletin No. 2 (Applied Biosystems, Foster City, CA, USA). The relative expression levels (fold changes) of mRNAs were calculated by $2-\Delta\Delta CT$ method. Real-time PCR products were normalized to their corresponding GAPDH mRNA.

### Protein analysis

Western blotting was performed by loading 30 µg protein on 8–12% (w/v) tris–glycine denaturing gels, separating proteins by electrophoresis, and then transferring to polyvinylidene difluoride (PVDF) membrane. After blocking, the membrane was incubated with primary antibodies; anti-phospho-IKKα/β (Ser176/180) (1:1000, #2697, Cell Signaling Technology), anti-NF-κB p65 (1:1000, #8242, Cell Signaling Technology), anti-occludin mouse monoclonal antibody (1:500, sc-133256, Santa Cruz), anti-cleaved caspase-3 rabbit monoclonal antibody (1:1000, #9664, Cell Signaling Technology), anti-cleaved caspase-9 rabbit monoclonal antibody (1:1000, #20,750, Cell Signaling Technology), anti-PARP-1 rabbit monoclonal antibody (1:1000, #9532, and Cell Signaling Technology at + 4 °C overnight. Following the incubation, the membrane was incubated with anti-β-actin mouse monoclonal antibody (1:1000, #3700, Cell Signaling Technology) at room temperature for 1 h. After washing, the membrane was incubated with peroxidase-conjugated secondary antibodies for 1 h to visualize labeled proteins by enhanced chemiluminescence. The analysis of the chemiluminescent blots was carried out with the aid of ChemiGlow detection reagents (Alpha Innotech). Subsequently, the visualization of protein bands was achieved using Fusion-FX7 (Vilber Lourmat, Thermo Fisher Scientific, US). Quantitative evaluation of these bands was done by employing the image analysis application ImageJ (version 1.48 s; National Institutes of Health). All tests were independently replicated for reliability. All proteins were assessed on the same membranes.

## Statistical analysis

All data were presented as means ± standard deviations (SD). The Shapiro–Wilk normality test was used to determine whether the data were normally distributed. Comparisons of means between two groups or multiple groups were performed by student *t*-tests or one-way analysis of

variance (ANOVA) followed by Tukey's post hoc test, respectively. The experiments were performed in 6 or 7 biological replicates with 3 technical replicates. *p*-values lower than 0.05 were regarded as statistically significant. Sample size calculations were performed by the power analyses program G*Power.

## Results

### Pioglitazone treatment decreases blood pressure in hypertensive rats

In order to evaluate whether pioglitazone has an effect on blood pressure in hypertensive rats, systolic blood pressure measurements were made with tail cuff plethysmography. Pioglitazone treatment significantly decreased systolic blood pressure from 148.5 to 136.5 mmHg in the BHR group; 151.5 to 142.0 mmHg in the SHR group ($p < 0.05$).

### Pioglitazone decreases proinflammatory cytokine levels and NF-κB activation

The possible effects of pioglitazone on the activation of proinflammatory cytokines and NF-kB in animal brainstem tissue samples in the study model were evaluated by ELISA and Western blot methods. In the present study, levels of both proinflammatory cytokines were found to be higher in the hypertension groups (Fig. 1). TNF levels in the BHR group were measured as 37.57 ± 13.44 ng/mg protein. It was observed that administration of PIO to the BHR group reduced TNF levels to 28.72 ± 3.70 ng/mg protein ($p < 0.05$) (Fig. 1a). Similarly, statistically significantly lower TNF levels were detected in PIO-treated SHR animals (10.68 ± 1.32 ng/mg protein vs. 27.82 ± 5.64 ng/mg protein, $p = 0.040$) (Fig. 1a).

IL-1β levels were measured at 60.99 ± 10.08 pg/mg protein in the SHR brainstem. It was observed that these values decreased to 33.01 ± 3.81 pg/mg protein levels following PIO treatment ($p = 0.0005$). In the BHR groups, statistically significantly lower IL-1β levels were measured in the PIO-treated

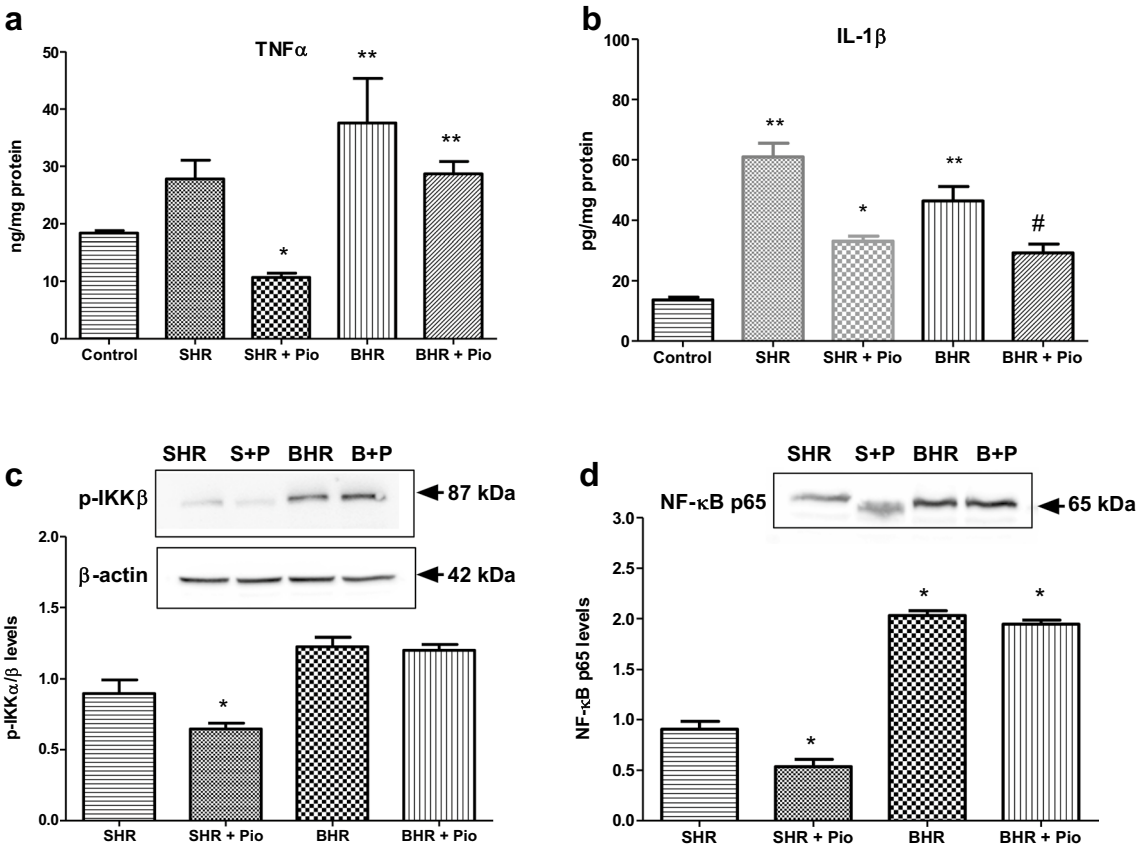


**Fig. 1** The levels of **a** TNFα, **b** IL-1β, **c** p-IKKβ, and **d** NF-κB p65 levels in brainstem of BHR and SHR groups following PIO treatment. Data were expressed as ng/mg protein for TNFα; pg/mg protein for IL-1β and reported as mean ± SD ($n = 6$). (*$p < 0.05$ vs. SHR group; **$p < 0.05$ vs. control group; #$p < 0.05$ vs. BHR; one-way ANOVA post-hoc Tukey) BHR, borderline hypertensive rats; SHR, spontaneously hypertensive rats; BHR + PIO, pioglitazone-treated BHR; SHR + PIO, pioglitazone-treated SHR

rats compared to the BHR group (46.34 ± 10.78 pg/mg protein vs. 29.22 ± 7.25 pg/mg protein, $p < 0.05$) (Fig. 1b).

In addition, Western blot analysis revealed activation of NF-κB in the brainstem of hypertensive rats, as indicated by the presence of NF-κB p65 and p-IKKβ (Fig. 1c and d). NF-κB activation was significantly decreased following PIO-treatment in the SHR group (Fig. 1d).

## Pioglitazone increases gene expression levels of tight junction proteins like occludin, claudin-2 and claudin-12

Similarly, the possible effects of pioglitazone on the mRNA levels of tight junction proteins were analyzed by Real-Time PCR method. When SHR and BHR groups were compared, no significant difference was found in gene expression levels between groups except for *claudin-2* and *NF-κB* (Fig. 2). C*laudin-2* and *NF-κB p65 subunit (Rela)* mRNA levels were found to be 7.50-fold and 4.76-fold higher in the BHR group when compared to the SHR group, respectively ($p = 0.001$).

In the PIO-treated SHR group; gene expression levels of *occludin*, *claudin-2*, and *claudin-12* were increased 8.27- ($p < 0.01$), 3.16- ($p < 0.01$), and 1.88-fold ($p < 0.05$), respectively, as compared to the SHR group (Table 1). Also, PIO treatment significantly downregulated *NF-κB p65 (Rela)* gene expression ($p < 0.05$) in the SHR group. However, there was no statistically significant difference in *claudin-1* and *ZO-1* gene expression in the SHR group ($p > 0.05$). As shown in Table 1, although PIO treatment tends to increase *occludin* gene expression in the BHR group, no statistically significant difference was found in gene expression between the BHR and PIO-treated BHR groups ($p > 0.05$).

## Pioglitazone increases protein levels of occludin and decreases cell death protein levels

Finally, the effects of pioglitazone on occludin and cell death proteins were investigated by PCR and Western blot analysis. Gene expression studies confirmed that occludin mRNA levels are higher in the PIO-treated SHR group. Occludin is

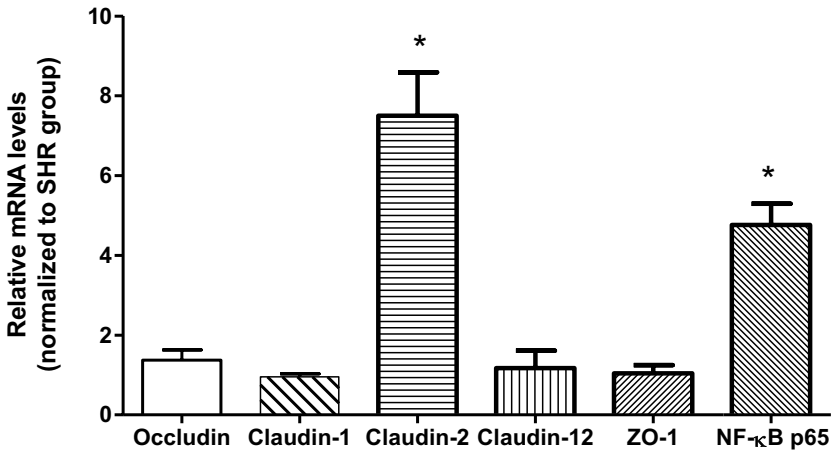


**Fig. 2** Relative quantification of *occludin*, *claudin-1*, *claudin-2*, *claudin-12*, *ZO-1*, and *NF-κB* p65 (Rela) mRNA levels in brainstem of BHR groups when normalized to SHR group. Real-time PCR products were normalized to its corresponding *GAPDH* mRNA. Data were reported as mean ± SD ($n = 3$). *$p < 0.01$ vs. SHR group; student's *t*-test

**Table 1** Relative fold changes in mRNA levels of selected genes; occludin, claudin-1, claudin-2, claudin-12, ZO-1, and NF-κB p65 (Rela) in brainstem of SHR and BHR groups following PIO treatment. Data were reported as mean ± SD ($n = 3$)

| Relative fold change | | | | | | |
|---|---|---|---|---|---|---|
| Gene | SHR | SHR + Pio | *p*-value (vs. SHR; Student's *t*-test) | BHR | BHR + Pio | *p*-value (vs.BHR; Student's *t*-test) |
| Occludin | 1.01 ± 0.19 | 8.27 ± 1.55 | 0.001 | 1.01 ± 0.19 | 1.47 ± 0.26 | ns |
| Claudin-1 | 0.99 ± 0.13 | 0.87 ± 0.03 | ns | 1.04 ± 0.18 | 1.04 ± 0.21 | ns |
| Claudin-2 | 1.00 ± 0.08 | 3.16 ± 0.21 | 0.001 | 1.00 ± 0.14 | 0.51 ± 0.06 | 0.046 |
| Claudin-12 | 1.01 ± 0.19 | 1.88 ± 0.31 | 0.049 | 1.05 ± 0.39 | 0.65 ± 0.13 | ns |
| ZO-1 | 1.02 ± 0.21 | 0.86 ± 0.10 | ns | 0.98 ± 0.19 | 1.23 ± 0.19 | ns |
| NF-κB p65 | 1.11 ± 0.18 | 0.52 ± 0.05 | 0.020 | 1.12 ± 0.11 | 1.06 ± 0.09 | ns |

*ns.* statistically nonsignificant, *BHR* borderline hypertensive rats, *SHR* spontaneously hypertensive rats, *Pio* pioglitazone

involved in tight junctions as well as intracellular signaling pathways, including apoptosis. Therefore, we investigated occludin protein levels as well. As shown in Fig. 3a and b, there were no statistically significant differences in occludin protein levels between the BHR and SHR groups. Occludin protein levels in the PIO-treated SHR group were 3.17-fold higher than in the SHR group ($p < 0.01$).

Caspase activation is an important step in the apoptotic cascade leading to cell death. Apoptosis is caused by various stimuli activating the enzymes caspase-9 and caspase-3,

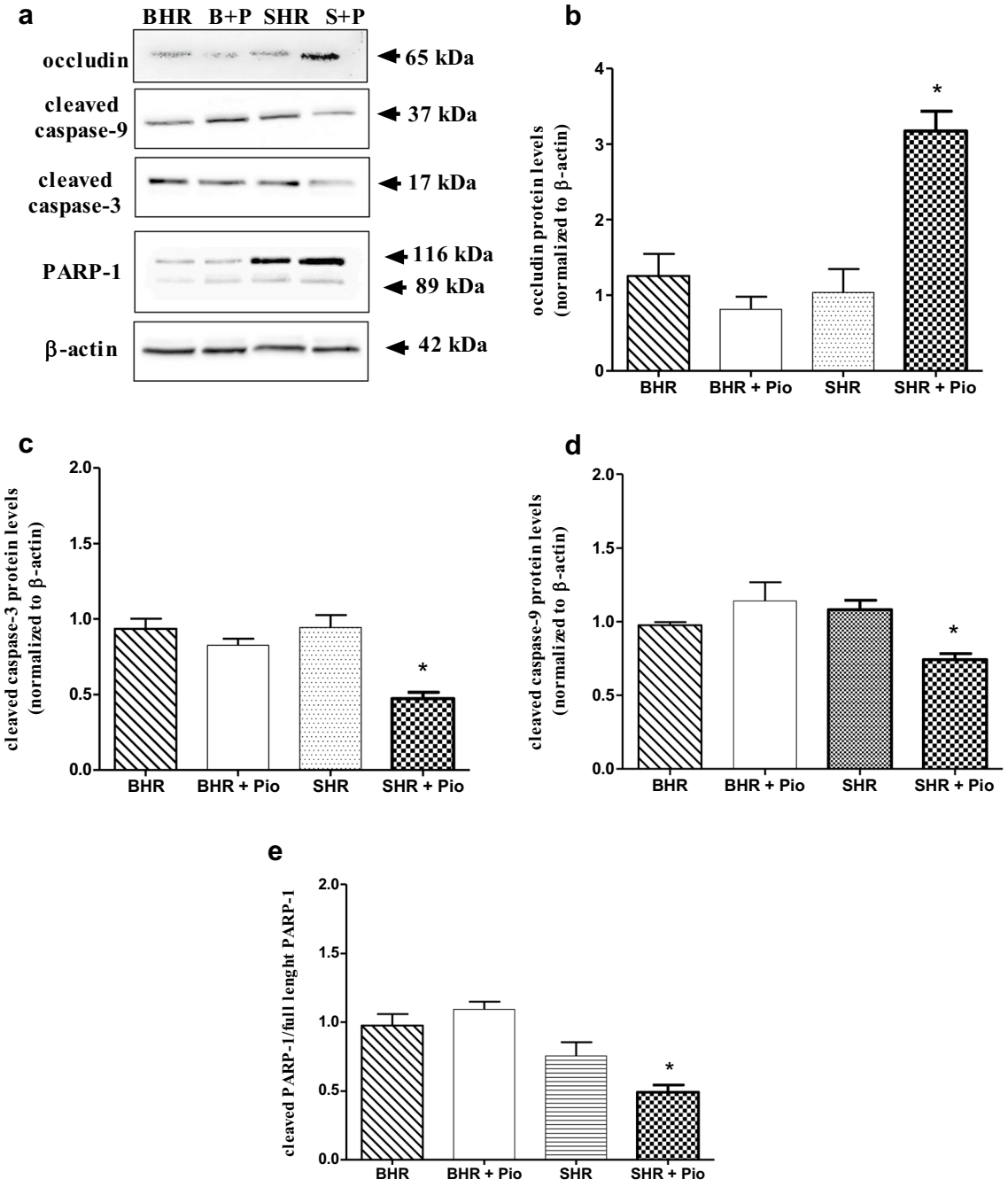


**Fig. 3** The protein expression levels of (**b**) occludin, (**c**) cleaved caspase-3, (**d**) cleaved caspase-9, and (**e**) PARP-1 in BHR and SHR groups following PIO treatment. (**a**) Protein expressions were determined by Western Blot analyses. Representative Western blots and graphs indicating the relative densitometric values of occludin, cleaved caspase-3, cleaved caspase-9, and PARP-1. Quantification of protein product was performed by densitometric scanning and data are normalized by using the β-actin signal. *$p < 0.01$ vs. SHR group; one-way ANOVA post-hoc Tukey. BHR, borderline hypertensive rats; SHR, spontaneously hypertensive rats; BHR + PIO, pioglitazone-treated BHR; SHR + PIO, pioglitazone-treated SHR

followed by PARP-1 cleavage. According to our findings, all three enzymes show a similar expression profile in the brainstem of untreated SHR and BHR groups. On the other hand, PIO treatment decreased the activation of caspases and cleavage of PARP-1, particularly in the SHR group. Western blot membrane image analysis confirmed that cleaved caspase-9, cleaved caspase-3, and cleaved PARP-1 levels were almost twofold lower than those of the SHR group in the PIO-treated SHR group (Fig. 3).

## Discussion

Our study sought to explore tight junction proteins, PPAR activity, inflammatory factors, and cell death proteins in the brainstems of spontaneously hypertensive rats (SHR) and borderline hypertensive rats (BHR). Upon treatment with pioglitazone, significant reductions were observed in the levels of pIKKβ, NF-κB p65, TNF, and IL-1β in the SHR group. Notably, the BHR group demonstrated higher expressions of key proteins such as occludin, claudin-2, claudin-12, and the NF-B p65 subunit compared to the SHR group. Pioglitazone's effectiveness in regulating gene expression was particularly discernible in the SHR group. The treatment led to an increase in the mRNA levels of occludin, claudin-2, and claudin-12, while a corresponding decrease was noted in cleaved caspase-3, caspase-9 levels, PARP-1 activation, and proinflammatory factor levels.

The above observations suggest that pioglitazone plays a critical role in regulating protein expression in hypertensive rat models. Its effect on mRNA levels, particularly for tight junction proteins, further highlights the drug's potential therapeutic relevance in the context of hypertension. The differential expressions of these proteins in SHR and BHR groups provide a promising avenue for future research to understand the underlying mechanisms in these hypertensive models.

The complexity and multiorgan effects of hypertension have led researchers to use animal models for understanding the pathogenesis and treatment strategies. Studying with the SHR model is of great importance because of its similarity with the pathophysiology of essential hypertension in humans and its use for screening drugs with potential antihypertension activities (Trippodo and Frohlich 1981; Zhu et al. 2016). As well as genetic models, psychogenic models such as BHR are preferred when studying hypertension induced by environmental stressors that increase sympathetic nervous system activity (Sanders and Lawler 1992).

Recent studies have shown that neuroinflammation in the brainstem contributes to the development of hypertension (Waki et al. 2007, 2008; Paton and Waki 2009; Wu et al. 2012; Winklewski et al. 2015). Experimental studies have also demonstrated the presence of oxidative stress and proinflammatory cytokines in the hypertensive brain (Guggilam et al. 2008; Gao et al. 2021). For this reason, the control of inflammation in the brainstem is an extremely valuable approach for both the regulation of blood pressure and neuronal protection.

In the current study, we compared the responses to PPARγ activation in terms of induction of cell death and inflammation in the brainstem with two experimental hypertension models. As shown in Fig. 1, the significant increases in proinflammatory mediators and NF-κB activation in hypertension models suggest neuroinflammation in the brainstem. High blood pressure and high cytokine levels are also consistent with those findings. It is speculated that the presence of pro-inflammatory cytokines affects adherents and tight junctions negatively (Winklewski et al. 2015; Rochfort et al. 2014). Several studies in intestine, kidney, heart, and brain tissue have reported that tight junction proteins decrease over time in hypertension (Wang et al. 2018; Santisteban et al. 2017). In particular, occludin and ZO-1 levels have been shown to change due to hypertension in various brain regions, including the hippocampus and cortex (Wang et al. 2018). When the BHR and SHR groups were compared, no significant difference was found in the gene expression profiles of tight junction proteins except claudin-2 in the brainstem (Fig. 2). Claudin-2 is a paracellular cation channel and facilitates the transition of cationic molecules by facilitating the formation of pores. It has been shown that increases in NF-κB, which is a factor associated with inflammation, are accompanied by a decrease in barrier-forming tight junction proteins but an increase in pore-forming claudin-2 (Günzel and Yu 2013).p-IKKβ is an important regulator of NF-kB activation. In this study, we examined changes in p-IKKβ and NF-B p65 protein levels to confirm NF-κB activation. The increase in p-IKKβ protein levels and NF-kB mRNA levels in hypertension models was found in accordance with upregulated proinflammatory cytokine levels and claudin-2 mRNA levels in the BHR group when compared to the SHR group. The activation of NF-kB may upregulate pore forming claudin-2 levels in the BHR model. The presence of severe inflammation could explain the higher claudin-2 mRNA expression levels in the BHR group in the current study.

PPARγ is a transcription factor whose physiological effects depend on its binding to a specific DNA sequence. PPARγ can lead to an increase or decrease in the transcription levels of target genes in a context-dependent manner. Disruption of these pathways can result in the development of several diseases and pathological states, such as obesity, obesity-induced inflammation, atherosclerosis, diabetes mellitus, metabolic syndrome, and hypertension. Tang et al. reported that hypertension development may be affected by renal PPARγ-dependent mechanisms (Tang et al. 2018). In several studies, the effects of PPARγ activators on blood pressure and related phenotypes such as endothelial function have been demonstrated (Kvandova et al. 2018; Grešová et al. 2019).

In our previous study, we confirmed that pioglitazone treatment regulates blood pressure and upregulates mRNA levels of PPARγ in the brainstem of SHR (Dovinová et al. 2013).

Thiazolidinediones (TZDs), including pioglitazone and rosiglitazone, are associated with a reduction in blood pressure in humans. The vasodepressor effects of TZDs are reported independent of the insulin-sensitizing properties of drugs, and using TZDs decreases cardiovascular risk by reducing mean systolic blood pressure of 3 mm Hg (Giles and Sander 2007). Similarly, a significant blood pressure decrease was observed in both SHR and BHR following pioglitazone treatment, which indicates its vasodepressor effects. In addition to their role in blood pressure regulation, the neuromodulatory functions of PPARγ agonists, particularly piogliazone, are the main interest of the current study.

Thus, to determine the genes affected as a result of PPARγ induction in hypertension, tight junction proteins were examined within the scope of the current study. In this paper, we hypothesized that PPARγ activation by pioglitazone reduces inflammation and neuronal death in the brainstem through regulating tight junction proteins during hypertension. As shown in Fig. 1, pioglitazone significantly regulated neuroinflammation in spontenous hypertensive rats, as evident by the significant decrease in IL-1β and TNF levels in brainstem tissues ($p < 0.05$). These results were in line with the observed mRNA expression changes of *occludin* and *claudin-12*. PPARγ modulation is shown to affect tight junction proteins in different cell types and animal models, including TBI, colitis, type 2 diabetes mellitus, nasal epithelial cells, etc. (Ogasawara et al. 2010; Min et al. 2012). Recently, Zhao et al. showed that rosiglitazone protects the blood–brain barrier by increasing occludin, ZO-1, and claudin-5 levels in the cerebral cortex in the traumatic brain injury model (Zhao et al. 2019). The above study supports our hypothesis that PPARγ activation affects tight junctions in brain tissue.

The elucidation of molecular mechanisms regulating neuronal death is of paramount importance for introducing appropriate therapeutic approaches to protect the brain during hypertension. Recently, there has been an increasing interest in the hypothesis that tight junction proteins could function as members of different signaling pathways that control gene expression, cell differentiation, and proliferation (Bednarczyk and Lukasiuk 2011). For example, occludin has been reported to affect many genes associated with apoptosis (Wang et al. 2018; Murata et al. 2005). Moreover, overexpression of claudin-1 has been shown to induce apoptosis in cancer cells (Poliak et al. 2002). Similarly, silencing of occludin in lung cancer cells is shown to promote caspase-3 and caspase-9 expression (Wang et al. 2018). On the contrary, Yu et al. showed that the number of apoptotic cells increased in kidney cell cultures upon silencing of occludin (Yu et al. 2005).

In this study, we examined tight junction proteins, PPAR activity, inflammatory factors, and cell death proteins in the brainstems of SHR and BHR. These two models were selected to represent different aspects of the pathophysiology underlying hypertension.

The SHR model represents a genetic model of hypertension and is widely used due to its similarity with the pathophysiology of essential hypertension in humans, as well as for drug screening for potential antihypertensive activities (Okamoto and Aoki 1963; Judy et al. 1976; Pinto et al. 1998).

On the other hand, the BHR model is particularly valuable in the context of the environmental aspect of hypertension. Recent studies have highlighted the important role of psychosocial stressors in the development and progression of hypertension. BHR is a unique model characterized by enhanced reactivity to stress, simulating the impact of environmental stressors on hypertension. In this regard, the BHR model fills a crucial gap in our understanding of the disease not fully captured by the SHR model. The choice of the BHR model was informed by the increasing recognition of the role of environmental stressors and psychosocial factors in the development of hypertension. Over the years, studies have highlighted the contribution of stress and other psychological factors to the onset and progression of hypertension, underscoring the need for animal models that adequately represent this complex interplay. BHR is one such model that demonstrates enhanced reactivity to stress. The increased stress reactivity in BHRs is thought to contribute to the progression from borderline to sustained hypertension, thereby bridging the gap between environmental stimuli and the physiological response in hypertension progression. Furthermore, our study delved into the neurobiological underpinnings of hypertension. We focused particularly on the brainstem proteins in these animal models, providing a detailed understanding of their roles under varying environmental and genetic influences. It's crucial to highlight that such neurobiological insights are instrumental in shaping our comprehension of hypertension and designing effective therapeutic strategies (D'Angelo et al. 2005; Julius et al. 1991; Gavras and Gavras 2002).

By comparing these two models, we aim to dissect the influence of genetic versus environmental factors on the pathogenesis of hypertension, particularly in relation to the activity of tight junction proteins, PPAR, and inflammatory markers in the brainstem. In this way, our study design allows for a comprehensive examination of the neurobiological underpinnings of hypertension under different genetic and environmental contexts. In summary, the selection of the BHR model for this study was strategically designed to emphasize the influence of psychological stressors on hypertension, which is an area not adequately represented in the SHR model. This research underlines the importance of incorporating psychosocial aspects into hypertension research and reinforces the significance of the BHR model as a viable complement to the SHR model in these investigations. In the current study, we have noted some key differences between the SHR and

**Table 2** Sequences of forward and reverse primers used for PCR analysis

| Gene | GeneBank number | Forward primer (5′→3′) | Reverse primer (5′→3′) | Base pair (bp) |
|---|---|---|---|---|
| Occludin | NM_031329.2 | TGACCAGTGACATCAGCCAT | ACGAGCATAGACAGGATCCG | 233 |
| Claudin 1 | NM_031699.2 | CTGTCGTTGGTGCTGTTTCA | CCTCTGCCCAACCTCAGTAA | 156 |
| Claudin 2 | NM_00110684.2 | GTGATTGAGGTGGGAGGACA | TGATGAGGTGTGCTGGGAAT | 160 |
| Claudin 12 | NM_001100813.1 | GCTGAGTGTTGGTCCTGTTG | ACCTGGCCCTGCTTTTATCT | 157 |
| ZO-1 | NM_001106266.1 | TATCCAAACCAGACCCACCC | GGCTTTGGTGTGAATCGGTT | 181 |
| NF-kB p65 subunit (Rela) | NM_199267.2 | CATCAAGATCAATGGCTACA | CACAAG TTCATGTGGATGAG | 94 |
| GAPDH | NM_017008.4 | AGACAGCCGCATCTTCTTGT | CTTGCCGTGGGTAGAGTCAT | 207 |

BHR models. Pioglitazone appears to effectively regulate gene expression in SHR, influencing levels of occludin, claudin-2, cleaved caspase-3, caspase-9, PARP-1 activation, and proinflammatory factor levels. On the other hand, we found the BHR group to express higher levels of occludin, claudin-2, claudin-12, and NF-B p65 subunit than the SHR group. The p-IKKβ, NF-κB p65, TNF, and IL-1β levels in the SHR group were also significantly reduced upon pioglitazone treatment.

Previous findings, combined with significant changes in occludin gene and protein expression in the SHR group (Table 2 and Fig. 3), suggest that pioglitazone can regulate caspase-3, caspase-9, and PARP-1 activity in our experimental models. As expected, a significant decrease in cleaved caspase-3, cleaved caspase-9, and cleaved PARP-1 in the brainstem of PIO-treated rats was observed in our models (Fig. 3). Collectively, it can be concluded that a caspase-dependent apoptotic pathway is triggered in hypertension and can be regulated by pioglitazone treatment in the hypertensive brain.

The brainstem is an important center for the regulation of blood pressure. Our data suggest that PPARγ activation by pioglitazone inhibits caspase-3 mediated intrinsic cell death in the hypertensive brain, and upregulation of occludin by PPARγ activation may have a role in emerging anti-apoptotic effects in the brainstem of essential hypertension. The limitation of the current preclinical study is the absence of an occludin-deficient model. Therefore, more research is needed to prove the role of occludin in the regulation of cell death during PPARγ activation. Also, in vitro and in vivo studies using specific inhibitors / activators should be conducted to clarify the link between tight junction proteins, PPARγ and neuroinflammation in hypertension.

## Conclusion

In conclusion, the present study investigated the effects of PPARγ activation on tight junction proteins and apoptosis in two experimental models of hypertension, BHR, and SHR. The findings of the study revealed that pioglitazone treatment decreased blood pressure, proinflammatory cytokine levels, and NF-κB activation in the brainstem of hypertensive rats. Pioglitazone treatment also regulated gene expression profiles of tight junction proteins and increased occludin protein levels in the SHR group. Furthermore, pioglitazone treatment inhibited caspase-3-mediated intrinsic cell death in hypertensive brain, and upregulation of occludin by PPARγ activation may have a role in emerging anti-apoptotic effects in the brainstem of essential hypertension. The present study highlights the potential therapeutic role of PPARγ activators in the management of hypertension, and further research is needed to elucidate the underlying molecular mechanisms and optimize treatment strategies.

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**Authors' contributions** NŞ and GA: conceived and designed research, wrote the manuscript, Conceptualization. NŞ, ID, DB, GK, MB, MAE and GA: Data curation, conducted experiments, NŞ, ID, DB, GK, MB, MAE and GA: Formal analysis, conducted experiments, edited the text. All authors read and approved the manuscript. The authors declare that all data were generated in-house and that no paper mill was used.

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**Data availability** The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Declarations

**Ethical approval** All animal experiments were approved by the Department of Animal Wellness, State Veterinary and Food Administration of the Slovak Republic (EÜHADYEK, 2019–071) 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.

**Competing interests** The authors declare no competing interests.

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