

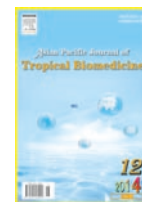
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Expression of p-PPAR γ in the aging thoracic aorta of spontaneously hypertensive rat and inhibitory effect of rosiglitazone

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ABSTRACT

Objective: To investigate the expression of phosphorylated peroxisome proliferators-activated receptor γ (p-PPAR γ) in the aging thoracic aorta of spontaneously hypertensive rat (SHR) and the inhibitory effect of rosiglitazone on the phosphorylation of PPAR γ .

Methods: 16, 32 and 64 week-old Wistar-Kyoto rats (WKY) and SHR were randomly and respectively divided into WKY, SHR and SHR+rosiglitazone group (9 in each group). The rats in SHR+rosiglitazone group were treated with rosiglitazone (5 mg/kg, intragastrically) for 56 d, whereas normal saline was applied in WKY and SHR groups. Systolic blood pressure (SBP) of rats was measured by tail cuff method. Histopathological damage of thoracic aorta was analyzed using Hematoxylin-Eosin (HE) staining. Immunohistochemical staining and western blot were performed to test the level of p-PPAR γ protein in the thoracic aorta arising from each group.

Results: The SBP in 16, 32 and 64 week-old SHR were significantly higher as compared with those in matched WKY rats ($P < 0.05$, respectively). HE staining showed increased content of smooth muscle cell, wrinkled lining endothelium and increased thickness of internal elastic lamina in the thoracic aorta of SHR. Immunohistochemical staining and western blot indicated that the levels of p-PPAR γ in the thoracic aorta arising from SHR were obviously higher than those in the thoracic aorta arising from WKY rats ($P < 0.05$, respectively). Importantly, the high SBP, histopathological abnormalities of the thoracic aorta and elevated p-PPAR γ expression were prominently abrogated by rosiglitazone treatment in SHR ($P < 0.05$, respectively). Furthermore, the SBP, histopathological abnormalities of the thoracic aorta and p-PPAR γ expression were positively correlated with age in SHR ($P < 0.05$, respectively).

Conclusions: The PPAR γ phosphorylation was observed in the thoracic aorta of SHR and its expression was increased by the increase of age. Furthermore, rosiglitazone inhibited the PPAR γ phosphorylation and suppressed vascular aging in SHR.

1. Introduction

Peroxisome proliferators-activated receptors γ (PPAR γ) is a ligands-activated nuclear transcription factor. Activated PPAR γ binds to the peroxisome proliferator responsive element in the

promoter of target gene to regulate gene expression[1,2]. For the past few years, PPAR γ has been considered as a well-known anti-aging molecule[3,4]. Activated PPAR γ plays a role in regulating adipocyte differentiation and lipid metabolism, improving insulin resistance, inhibiting inflammatory reaction[5] and up-regulating longevity gene expression such as Klotho[6]. Previous studies reported that PPAR γ inactivation by phosphorylation was observed in many kinds of tissues, such as kidney[7], cerebral cortex[8] and fat[9], suggesting that PPAR γ phosphorylation may play an important role in the initiation and development of aging and leads to functional disorder and aberrant expression

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of downstream target gene. Vascular aging can change the threshold and severity of cardiovascular diseases including hypertension[10]. It is an independent risk factor for the increasing incidence of hypertension with older age[10,11]. However, whether PPAR γ inactivation and the degree of PPAR γ phosphorylation are presented during hypertension related vascular aging remains undetermined.

Recently, Choi *et al.* reported that cyclin-dependent kinase 5 (Cdk5) could lead to PPAR γ phosphorylation in adipocytes and subsequently result in functional disorder and aberrant expression of obesity related gene. PPAR γ phosphorylated by Cdk5 is inhibited by rosiglitazone, a PPAR γ agonist, *in vitro* and *in vivo*[12]. Furthermore, the inhibitory effect is independent of canonical receptor transcriptional activation pathway. These results indicate that rosiglitazone may inhibit PPAR γ phosphorylation in vascular aging of hypertension patients. In this study, 16, 32 and 64 week-old spontaneously hypertension rats (SHR) were treated with rosiglitazone. We aimed to investigate PPAR γ phosphorylation in the thoracic aorta of SHR during vascular aging and explore the inhibitory effect of rosiglitazone on PPAR γ phosphorylation.

2. Materials and methods

2.1. *In vivo* experiments

The 16, 32 and 64 week-old SHR ($n=54$) and Wistar-Kyoto (WKY) rats ($n=27$) were purchased from Shanghai Slack Laboratory Animal Co., LTD (Permit number: SCXK: 2007-0005, Shanghai, China) and cultured in the Centre of Laboratory Animals, Medical College of Xi'an Jiaotong University. Rats were housed in sterilized cages (2 rats/cage) at a constant temperature (23 °C) and humidity and fed a regular autoclaved diet with water *ad libitum*[13]. Rats were respectively divided into WKY ($n=9$), SHR ($n=9$) and SHR+rosiglitazone groups ($n=9$). Rats in the SHR+rosiglitazone group were treated with rosiglitazone (5 mg/kg) daily for 8 weeks through intragastric administration. While, rats in the WKY and SHR groups were treated with the same volume of saline. Blood pressure measurement was performed after treatment. After treatment, systolic blood pressure (SBP) was measured using tail cuff method as previous described[14]. All rats were sacrificed by cervical vertebra dislocation under anesthesia with ether and the thoracic aorta tissues were excised and prepared for routine pathological examination with Hematoxylin-Eosin (HE) staining[13]. Some thoracic aorta tissues ($n=3$) were subjected to immunohistochemical analysis, and some ($n=6$) were homogenized and the proteins extracted for immunoblotting. All animal protocols were approved by the Institutional Animal Care and Use Committee of Xi'an Jiaotong University.

2.2. Immunohistochemical staining

Immunohistochemistry was performed on paraformaldehyde-

fixed paraffin sections with streptavidin peroxidase conjugated method to detect the positive expression of phosphorylated PPAR γ (p-PPAR γ) in the aortas of rats according to the manufacturer's instructions (Zhongshan Golden Bridge Biotechnology Ltd. Co., Beijing, China). The sections of thoracic aorta were routinely dewaxed and antigen retrieval in citrate buffer (0.01 mol/L, pH 6.0). The sections were subsequently blocked using 10% goat plasma and incubated using p-PPAR γ (Ser 273; Beijing Bioss Biological Technology Ltd. Co., Beijing, China) (1:600) primary antibody at 4 °C overnight. Biotinylated secondary antibodies were used to detect the primary antibody. The sections were visualized with diaminobenzidine (Wuhan Boster Biological Engineering Ltd. Co., Wuhan, China) and counterstained with hematoxylin, then dehydrated in alcohol and xylene and mounted onto glass slides. Phosphate buffer solution was used as primary antibody for negative control.

2.3. Western blot

The total proteins were isolated from thoracic aorta and measured using Protein Quantitative Reagent Kit-BCA Method (Beijing Bioss Biological Technology Ltd. Co.). After protein denaturation at 95 °C, proteins were separated using 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred into the nitrocellulose filter. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (G8140; US Biological, Swampscott, MA, USA) (1:5 000) and p-PPAR γ (1:800) primary antibodies were used in the immunoblotting assays. Horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit secondary antibodies (Bio-Rad, Hercules, CA, USA) were used at a 1:1 000-1:5 000 dilution and detected using a Western Blotting Luminol Reagent (sc-2048; Santa Cruz Biotechnology), as described in previous study[15].

2.4. Statistical analysis

The data were analyzed using the SPSS statistical package for Windows Version 13 (SPSS, Chicago, IL, USA). Results were expressed as mean \pm SEM. Levene test and Shapiro-Wilk test were performed to determine the homogeneity test for variance and normality test, respectively. Significance was established using One-way ANOVA and LSD-*t* test when appropriate. Difference was considered significant when $P<0.05$.

3. Results

3.1. The SBP of SHR and WKY rats with different age

As shown in Figure 1, the SBP in 16, 32 and 64 week-old SHR were significantly higher than those in matched WKY rats ($P<0.05$). However, the SBP in rosiglitazone treated SHR were obviously lower as compared with those in matched SHR

($P < 0.05$). In SHR, 64 week-old rats showed the highest SBP as compared with either 32 week-old or 16 week-old rats ($P < 0.05$, respectively). But 16 week-old SHR exhibited the lowest SBP as compared with 32 week-old rats ($P < 0.05$). Comparison of SBP in 16, 32 and 64 week-old WKY rats, the highest SBP was observed in 64 week-old rats ($P < 0.05$, respectively, Figure 2), while 16 week-old rats showed the lowest SBP ($P < 0.05$, respectively, Figure 2).

3.2. The structure of thoracic aorta in SHR and WKY rats with different age

As shown in Figure 3, HE staining showed the layer of elastic fibers was no big change, but exhibited increased content of smooth muscle cell, wrinkled lining endothelium and increased

thickness of internal elastic lamina in the thoracic aorta of SHR. These histopathological changes were worsened by the increase of age. The histopathological changes in SHR+rosiglitazone group were less than those in SHR group. WKY group only showed increased layer of elastic fibers with the increase of age. But other histopathological changes were not observed in WKY group.

3.3. The expression of p-PPAR γ in the thoracic aorta of rats

The thoracic aortas arising from rats in different groups were subjected to immunostaining for p-PPAR γ . As shown in Figure 4, our results found that p-PPAR γ signal was increased by the increase of age in both SHR and WKY rats. P-PPAR γ signal in SHR group was obviously higher than that in matched WKY group. However, SHR+rosiglitazone group showed less p-PPAR γ signal

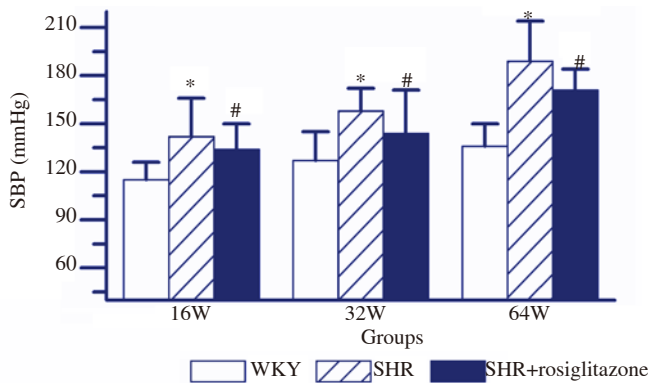


Figure 1. SBP in different groups with different age. The SBP in SHR group was significantly higher than that in WKY group. However, The SBP in SHR+rosiglitazone group was obviously lower than that in SHR group. Values are depicted as mean \pm SEM, $n=9$. * $P < 0.05$ vs WKY group; # $P < 0.05$ vs SHR group.

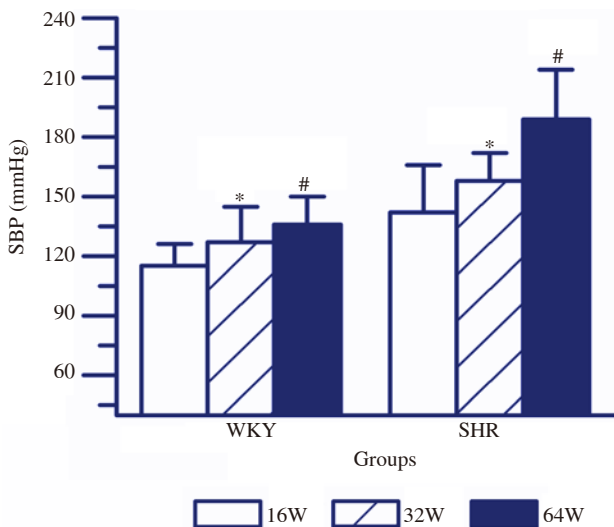


Figure 2. SBP in SHR and WKY rats with different age. The SBP in 64 week-old SHR was prominently higher than that in either 32 or 16 week-old rats. While 16 week-old SHR showed the lowest SBP. Similarly, The SBP in 64 week-old WKY rats was significantly higher than that in either 32 or 16 week-old rats. While 16 week-old WKY rats showed the lowest SBP. Values are depicted as mean \pm SEM, $n=9$. * $P < 0.05$ vs 16 week; # $P < 0.05$ vs 32 week.

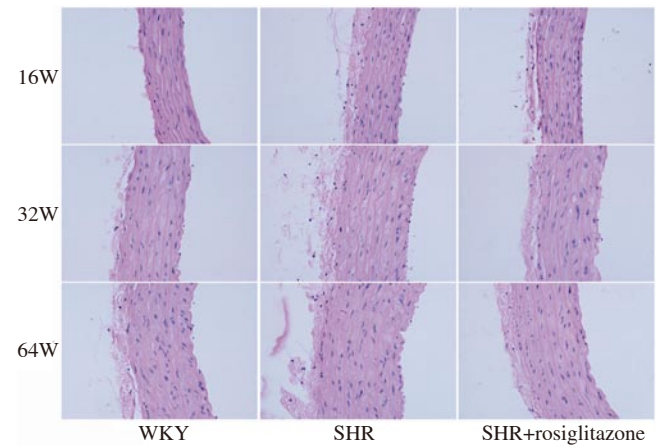


Figure 3. HE staining of the thoracic aorta of rats in different groups. The histopathological changes were worsened by the increase of age in SHR group. The histopathological changes in SHR+rosiglitazone group were less than those in SHR group. However, WKY group showed slight histopathological changes. Original magnification $\times 100$.

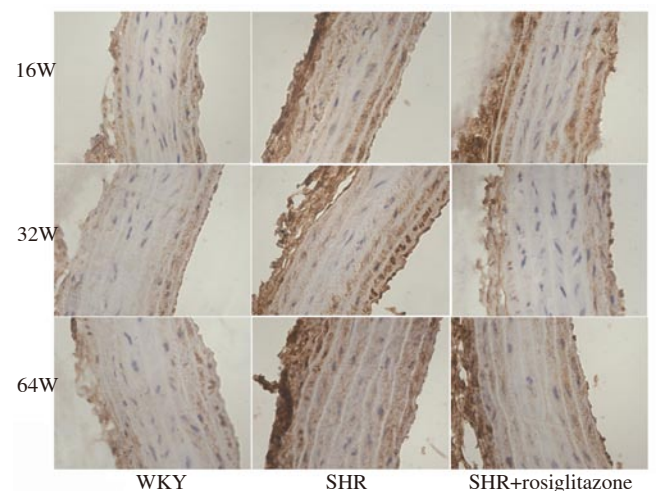


Figure 4. Immunohistochemical analyses of p-PPAR γ in the thoracic aorta of rats in different groups. The p-PPAR γ signal was up-regulated by the increase of age in both SHR and WKY groups. The p-PPAR γ signal in SHR group was higher than that in WKY group. However, SHR+rosiglitazone group showed a weak p-PPAR γ signal as compared with SHR group. Original magnification $\times 400$.

as compared with SHR group. As shown in Figures 5A and 5B, western blot analysis indicated that the levels of p-PPAR γ in the thoracic aorta isolated from 16, 32 and 64 week-old SHR were significantly higher than those in matched WKY rats ($P < 0.05$, respectively). Whereas, the levels of p-PPAR γ in the thoracic aorta arising from rosiglitazone treated SHR were obviously lower than those in matched SHR ($P < 0.05$, respectively). In SHR, 64 week-old rats showed the highest p-PPAR γ expression as compared with either 32 week-old or 16 week-old rats ($P < 0.05$, respectively). But 16 week-old SHR exhibited a lower p-PPAR γ expression as compared with 32 week-old rats ($P < 0.05$). Comparison of p-PPAR γ expression in 16, 32 and 64 week-old WKY rats, the highest p-PPAR γ expression was observed in 64 week-old rats ($P < 0.05$, respectively, Figure 5B), while 16 week-old rats showed the lowest p-PPAR γ expression ($P < 0.05$, respectively, Figure 5B).

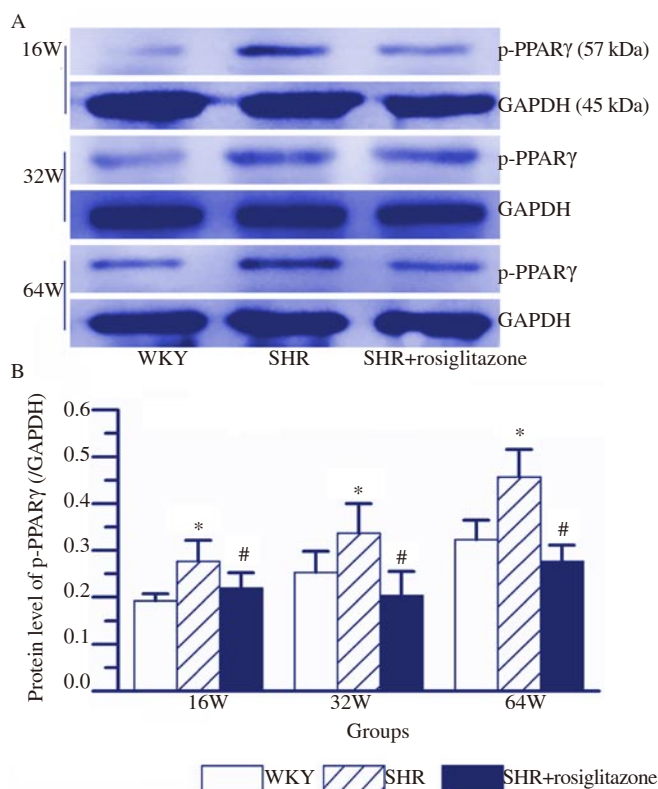


Figure 5. Western blot analysis of p-PPAR γ protein in the thoracic aorta of rats in different groups.

A: Representative western blot analysis of p-PPAR γ expression in the thoracic aorta of rats in different groups; B: Quantification of the data revealed that p-PPAR γ expression in SHR group was higher than that in WKY group. However, SHR+rosiglitazone group showed a less p-PPAR γ level as compared with SHR group. Values are depicted as mean \pm SEM, $n=6$. * $P < 0.05$ vs WKY group; # $P < 0.05$ vs SHR group.

4. Discussion

Vascular aging is mainly characterized as characteristic remodeling of the structure and function by the increase of age, which is an independent risk factor for promoting the incidence

of hypertension[10,11]. In this study, SBP were measured using a tail cuff method. Our data indicated that the SBP in SHR was elevated by the increase of age. HE staining showed that the histopathological changes including increased content of smooth muscle cell, wrinkled lining endothelium and increased thickness of internal elastic lamina in the thoracic aorta of SHR were worsened by the increase of age. These results indicate that vascular aging is observed in the thoracic aorta of SHR. PPAR γ acts as a nuclear transcription factor for regulating several senescence-associated genes[3-6]. PPAR γ phosphorylation is an important post-transcriptional modification and it has been found in many aging diseases[7-9]. However, whether PPAR γ phosphorylation is presented in vascular aging induced hypertension has not been investigated. We, for the first time, approved that p-PPAR γ was observed in the thoracic aorta of 16, 32 and 64 week-old SHR by the increase of age, which was consistent with vascular aging in the thoracic aorta. These results suggest that PPAR γ phosphorylation is enhanced during hypertension related vascular aging. But the priority of PPAR γ phosphorylation or vascular aging needs to be further confirmed.

Different phosphorylated residues of PPAR γ in different cells and under different stimulations exert different biological effects[16]. Mitogen-activated protein kinases induce the phosphorylation of PPAR γ on Ser112 and inhibit PPAR γ activity[17-19]. However, cyclin-dependent kinase 7 (Cdk7) and Cdk9 lead to phosphorylation of PPAR γ on Ser112 and increase PPAR γ activity[20,21]. Otherwise, Cdk5 mediates Ser273 phosphorylation, which is located at hinge region between PPAR γ DNA binding domain and ligand binding domain, to regulate target gene expression[12]. Our studies just detected the level of p-PPAR γ with Ser273 phosphorylation. Thus, the level of p-PPAR γ with Ser112 phosphorylation still needs further studies. Previous studies have approved that PPAR γ agonist, rosiglitazone, inhibits Cdk5 mediated phosphorylation of PPAR γ on Ser273 in adipocytes and is independent of canonical receptor transcriptional activation signaling pathway[12]. Our studies further confirmed that rosiglitazone inhibits the phosphorylation of PPAR γ on Ser273 in the thoracic aorta of SHR. But further researches are needed to confirm that the effect is mediated by Cdk5 pathway or not. Otherwise, PPAR γ functions as a nuclear transcription factor for regulating many target gene expression. Whether rosiglitazone binds to PPAR γ and regulates phosphorylation related target gene expression to inhibit PPAR γ phosphorylation still need to be approved.

In conclusion, PPAR γ phosphorylation is observed in the thoracic aorta of SHR during vascular aging. Furthermore, the level of p-PPAR γ protein is up-regulated by the increase of age. Rosiglitazone inhibits PPAR γ phosphorylation and vascular aging. These results contribute to revealing the mechanisms involved in hypertension vascular aging.

Conflict of interest statement

We declare that we have no conflict of interest.

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