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Original Article

SHH signaling mediates the effect of PPAR β/δ on angiogenesis following cerebral ischemia

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Abstract: Aims: This study aimed to investigate whether Sonic Hedgehog (SHH) mediates the effects of peroxisome proliferator-activated receptor beta/delta (PPAR β/δ) on angiogenesis following cerebral ischemia. Methods: A rat middle cerebral artery occlusion (MCAO) model was established. Ischemic stroke was stimulated *in vitro* by performing oxygen-glucose deprivation (OGD) on human umbilical vein endothelial cells (HUVECs). The activation of PPAR β/δ was induced by treatment with GW501516. The expression of von Willebrand factor (vWF), SHH, vascular endothelial growth factor A (VEGFA) and Notch1 were examined by western blot analysis. Cell proliferation, cell migration and tube length were determined by MTT, Transwell-migration and tube formation assays. SHH knockdown was achieved by transfection of SHH-siRNA. Results: PPAR β/δ activation induced angiogenesis in the infarct cerebral hemisphere following MCAO, as demonstrated by up regulated expression of vWF. PPAR β/δ activation also increased the expression of SHH, VEGFA and Notch1 in the MCAO rats. The results from *in vitro* studies showed that SHH expression was induced by GW501516 treatment in OGD-treated HUVECs. PPAR β/δ activation induced angiogenesis under OGD condition, as demonstrated by increased cell proliferation, cell migration and tube formation; however, these effects were attenuated by SHH knockdown. SHH knockdown also attenuated the effect of PPAR *β/δ on* VEGFA and Notch1 expression. Conclusion: This study firstly demonstrated that PPAR β/δ activates SHH signaling in cerebral ischemia, and SHH mediates the effect of PPAR β/δ on angiogenesis. The regulation of PPAR β/δ/SHH/VEGFA/Notch1 pathway may be a novel therapeutic intervention for cerebral ischemia.

Keywords: Peroxisome proliferator-activated receptor beta/delta, Sonic Hedgehog, cerebral ischemia, angiogenesis

Introduction

Stroke causes irreversible brain damage that may lead to progressive dementia and cognitive deterioration. Good collateral circulation could improve the clinical prognosis of stroke [1]. Angiogenesis is the formation of new capillaries from pre-existing vessels [2], and it has become an effective method to prevent brain injury following ischemia [3].

Peroxisome proliferator-activated receptors (PPARs) belong to the nuclear hormone receptor super family [4]. They are ligand-activated transcription factors and play pivotal roles in the pathogenesis of various disorders [5-7]. Three different PPAR isoforms, including alpha, beta/delta, and gamma, have been identified [8]. PPAR β/δ is the most ubiquitously expressed PPAR [9, 10]. However, the physiological function of PPAR β/δ has been explored limitedly. Recently, some researchers reported that PPAR β/δ activation protects against acute ischemic stroke in animal models and induces endothelial cell proliferation and angiogenesis [11, 12]. It has been suggested that the biological effects induced by activation of PPAR β/*δ* are mediated through some potential pathways [13]. Sonic Hedgehog (SHH) signaling is a well-known pathway which involves in the pathogenesis of brain ischemia [14-17].

In the present study, we firstly investigated whether SHH signaling mediates the effects of PPAR β/δ on angiogenesis following cerebral ischemia.

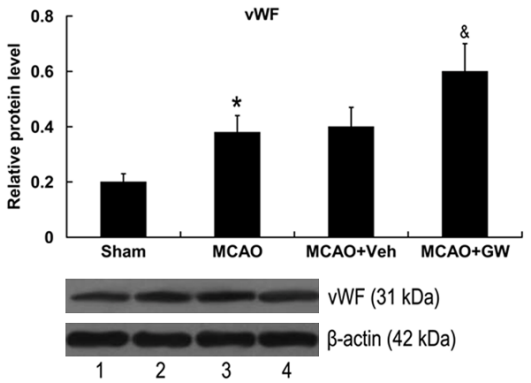

Figure 1. Expression of vWF protein in the infarct cerebral hemisphere. Lane 1, sham; lane 2, MCAO; lane 3, MCAO+Veh; lane 4, MCAO+GW. $^{*}P<0.05$ vs. sham; $^{\&}P<0.05$ vs. MCAO+Veh. vWF, von Willebrand factor; MCAO, middle cerebral artery occlusion; Veh, vehicle; GW, GW501516.

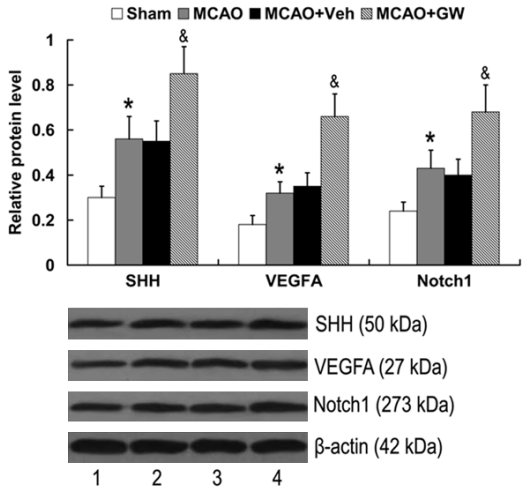

Figure 2. Expression of SHH, VEGFA and Notch1 proteins in the infarct cerebral hemisphere. Lane 1, sham; lane 2, MCAO; lane 3, MCAO+Veh; lane 4, MCAO+GW. $^{*}P<0.05$ vs. sham; $^{\&}P<0.05$ vs. MCAO+Veh. SHH, Sonic Hedgehog; VEGFA, vascular endothelial growth factor A; MCAO, middle cerebral artery occlusion; Veh, vehicle; GW, GW501516.

Materials and methods

Construction of rat middle cerebral artery occlusion (MCAO) model

The male Sprague-Dawley rats 6-8 weeks old were obtained from Shanghai Slac Laboratory Animal Company (Shanghai, China). This study was approved by the Ethics Committee of 148 Hospital of PLA, and all procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals (NIH Publication no. 86-23, revised 1986). 32 rats were divided into four groups randomly: sham group (n=8), MCAO group (n=8), MCAO+vehicle group (n=8) and MCAO+GW501506 group (n=8). 100 μL GW501516 (10 μg/μL; Axxora Biochemicals, San Diego, CA, USA) or the vehicle control artificial cerebrospinal fluid (ACSF) were injected to the lateral ventricle of the rats 24 h before MCAO experiments. A MCAO model was performed according to the study by Longa et al with some modifications [18]. The rats were anesthetized with 10% chloral hydrate (4 ml/kg; Shuanghe Limited, Beijing, China) intraperitoneally, and placed in the supine position. The right common carotid artery was exposed following a 2-cm midline incision in the neck. Then, a 4-0 nylon suture coated with a silicone tip was inserted into the internal carotid artery to occlude the middle cerebral artery. The rats in the sham group were subjected to surgery without the occlusion of the middle cerebral artery. The neurological function was tested using the method described by Longa et al [18], and rats with the scores of 1-3 were included. 24 h following MCAO, the tissues in the infarct cerebral hemisphere were collected.

Cell culture

Human umbilical vein endothelial cells (HUVECs) were purchased from the American Type Culture Collection (Baltimore, MD, USA). Cells in normal condition were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen) in a 5% CO_2, 95% air atmosphere with constant humidity. For oxygen-glucose deprivation (OGD)-treated cells, the HUVECs were grown in DMEM without glucose, in a 5% CO_2, 95% N_2 atmosphere for 10 h. Then, the medium was replaced by fresh DMEM supplemented with 10% FBS, and maintained in a 5% CO_2, 95% air atmosphere. GW501516 was diluted into 100 nM and treated the cells for 24 h.

Cell transfection

SHH siRNA was transfected into the cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. 50 pmol of siRNA was diluted in 250 μL serum-free Opti-MEMI (Invitrogen), and combined with 5 μL of Lipofectamine 2000 in 250 μL serum-free

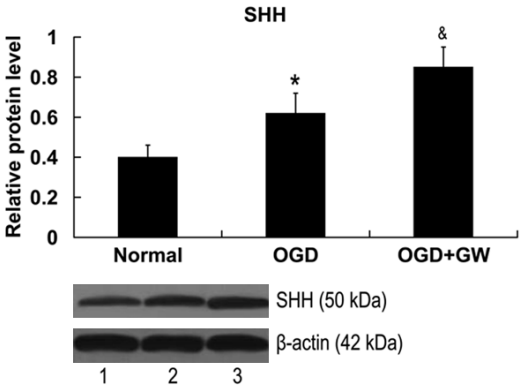

Figure 3. Expression of SHH protein in HUVECs. Lane 1, normal; lane 2, OGD; lane 3, OGD+GW. $^{*}P<0.05$ vs. normal; $^{\&}P<0.05$ vs. OGD. SHH, Sonic Hedgehog; OGD, oxygen-glucose deprivation; GW, GW501516.

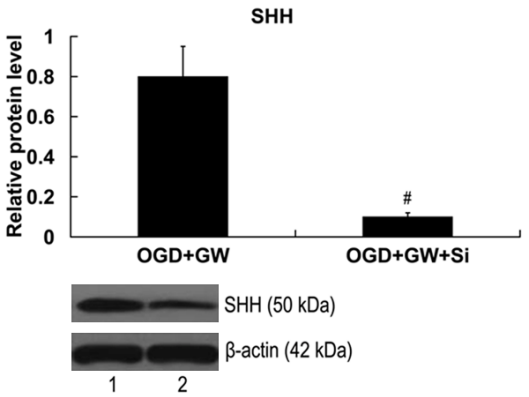

Figure 4. Expression of SHH protein in HUVECs transfected with SHH-siRNA. Lane 1, OGD+GW; lane 2, OGD+GW+Si. $^{\#}P<0.01$ vs. OGD+GW. SHH, Sonic Hedgehog; OGD, oxygen-glucose deprivation; GW, GW501516; Si, SHH-siRNA.

OPTI-MEMI. After incubation at room temperature for 20 min, the mixture was applied to each well, and incubated at 37°C for 6 h. The cultures were then replaced with the fresh medium.

MTT assay

Cell proliferation was assessed using MTT assay. The cells were plated on 96-well plates at the density of 1×10^4 cells in 100 μL medium per well, and allowed to grow for appropriate times. 10 μL of MTT solution (0.5 mg/ml; Sigma, St. Louis, MO, USA) was added into each well. After incubation at 37°C for 4 h, 150 μL of DMSO (Sigma) was added into each well to dissolve the formazan crystals. Viable cells were measured by absorbance at 570 nm by a microplate reader.

Transwell migration assay

The transwell migration assay was performed using 6-well Transwell plates (8 μm; Corning, Inc., Corning, NY, USA). The cells were washed and resuspended in DMEM without FBS at the density of 5×10^4 cells/ml. 2 ml of cell suspension was placed in the upper chambers. The lower chambers were filled with 1 mL DMEM containing FBS. After incubation at 37°C for 24 h, the non-migrated cells in the upper chambers were removed by a cotton swab, the migrated cells were fixed in 95% methanol and stained with hematoxylin. Under the microscope, six random fields were selected to analyze the number of migrated cell of each chamber.

Tube formation assay

200 μL of BD Matrigel matrix (BD Biosciences, Franklin Lakes, NJ, USA) was added to 24-well plate, and then incubated at 37°C for 30 min to allow gel formation. The cells were washed and resuspended at the density of 4×10^5 cells/mL. 50 μL of cell suspension was placed in each well and the medium was compensated to 1 mL. After incubation at 37°C for 8 h, the tube formation was observed under the microscope and imaged. Ten random fields were selected to analyze the tube lengths using Image-Pro Plus 5.0 (BD Biosciences) software.

Western blot

The tissues and cells were lysed in radioimmunoprecipitation assay buffer (Beyotime, Shanghai, China). After centrifugation at 13,000 g for 1 min, a total of 50 μg lysates were fractionated on a 10% SDS polyacrylamide gel and electrophoretically transferred onto nitrocellulose membranes (EMD Millipore Corporation, Billerica, MA, USA). The membranes were blocked with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) overnight at 4°C. The primary antibodies, including mouse monoclonal to vWF, mouse monoclonal to SHH, mouse monoclonal to VEGFA and mouse monoclonal to Notch1 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Mouse monoclonal to

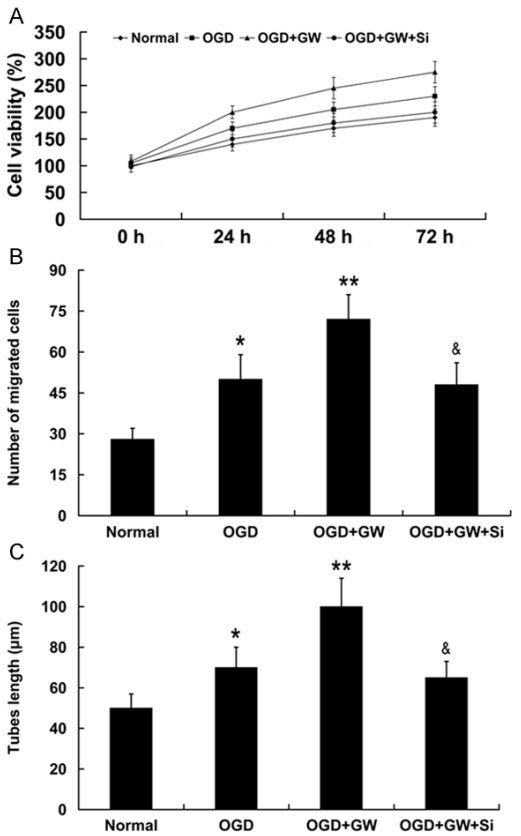

Figure 5. SHH suppression attenuated the effect of PPAR β/δ on angiogenesis in OGD-treated HUVECs. A. Cell proliferation determined by MTT assay. B. The number of migrated cell determined by Transwell-migration assay. C. Tube length of HUVECs determined by tube formation assay. $^{*}P<0.05$ vs. normal; $^{**}P<0.05$ vs. OGD; $^{\&}P<0.05$ vs. OGD+GW. OGD, oxygen-glucose deprivation; GW, GW501516; Si, SHH-siRNA.

β-actin was purchased from Boster (Wuhan, Hubei, China). After washing with PBS, the membranes were incubated with the primary antibodies at 4°C overnight. This was followed by incubation with goat anti-mouse horseradish peroxidase-conjugated antibody (Boster) at 37°C for 1 h. Detection of the signals was accomplished by using the chemiluminescence detection system (ECL western blotting kit; Pierce Biotechnology, Inc. Rockford, IL, USA). β-actin was used as an internal control.

Statistical analyses

The data are expressed as mean ± SD. Comparisons between 2 groups were made using the Student's t-test. Significance was considered at a value of $P<0.05$.

Results

PPAR β/δ activation induced angiogenesis in the infarct cerebral hemisphere following MCAO

The expression of the endothelial cell surface marker von Willebrand factor (vWF) was examined to determine angiogenesis in the infarct cerebral hemisphere. We found that vWF protein was significantly up regulated in the infarct cerebral hemisphere following MCAO.

To investigate the effect of PPAR β/δ activation on angiogenesis following MCAO, the rats in the MCAO+GW group were pretreated with PPAR β/δ ligand GW501516 24 h before MCAO. The rats in the MCAO+Veh group were pretreated with ACSF. As shown in **Figure 1**, compared with the MCAO group, the expression of vWF protein did not alter in the infarct cerebral hemisphere in the MCAO+Veh group; however, vWF expression was significantly increased in the MCAO+GW group.

PPAR β/δ activation induced the expression of SHH, vascular endothelial growth factor A (VEGFA) and Notch1 in the infarct cerebral hemisphere following MCAO

The expression of SHH, VEGFA and Notch1 in the infarct cerebral hemisphere were examined by western blot analysis. As shown in **Figure 2**, compared with the sham group, the relative protein levels of SHH, VEGFA and Notch1 were significantly upregulated in the MCAO group. We found that pretreatment with ACSF did not show any effect on the expression of SHH, VEGFA and Notch1 protein; however, compared with the rats pretreated with ACSF, pretreatment with GW501516 significantly increased the relative protein levels of SHH, VEGFA and Notch1 in the infarct cerebral hemisphere following MCAO.

PPAR β/δ activation induced the expression of SHH in OGD-treated HUVECs

The HUVECs were exposed to OGD condition to establish the *in vitro* model of ischemia. As demonstrated in **Figure 3**, the relative protein level of SHH in the OGD-treated HUVECs was higher than that in the normal HUVECs. Furthermore, we found that SHH expression was induced in OGD-treated HUVECs by GW501516 treatment.

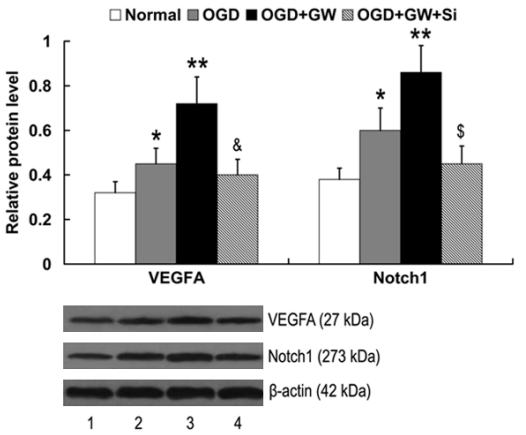

Figure 6. Expression of VEGFA and Notch1 proteins in HUVECs transfected with SHH-siRNA. Lane 1, normal; lane 2, OGD; lane 3, OGD+GW; lane 4, OGD+GW+Si. $^{*}P<0.05$ vs. normal; $^{**}P<0.05$ vs. OGD; $^{\&}P<0.05$, $^{\$}P<0.01$ vs. OGD+GW+Si. VEGFA, vascular endothelial growth factor A; OGD, oxygen-glucose deprivation; GW, GW501516; Si, SHH-siRNA.

SHH suppression attenuated the effect of PPAR β/δ on angiogenesis in OGD-treated HUVECs

To investigate whether SHH mediates the effect of PPAR β/δ on angiogenesis in OGD-treated HUVECs, SHH protein was knocked down by siRNA, then cell proliferation, migration and tube formation were examined. As shown in **Figure 4**, GW501516-induced expression of SHH was significantly suppressed by SHH-siRNA in OGD-treated HUVECs. Subsequently, it was revealed that compared with the normal HUVECs, OGD-treated HUVECs showed increased ability of cell proliferation, migration and tube formation. GW501516 treatment further induced cell proliferation, migration and tube formation of OGD-treated HUVECs; however, these effects of GW501516 on angiogenesis was attenuated by SHH knockdown. The cell viability, number of migrated cells and tubes length were significantly decreased in the OGD+GW+Si group compared with the OGD+GW group (**Figure 5**).

SHH suppression attenuated the effect of PPAR β/δ on VEGFA and Notch1 expression in OGD-treated HUVECs

Western blot analysis was used to investigate whether SHH mediates the effect of PPAR β/δ on VEGFA and Notch1 expression in OGD-treated HUVECs. As shown in **Figure 6**, the expression of VEGFA and Notch1 was increased in HUVECs under OGD condition. GW501516 treatment significantly up regulated VEGFA and Notch1 expression. However, SHH knockdown attenuated the inductive effect of GW501516 on VEGFA and Notch1 expression, and the relative protein levels of VEGFA and Notch1 were significantly decreased in the OGD+GW+Si group compared with the OGD+GW group.

Discussion

In the study by Chao et al [11], it has been demonstrated that the PPAR β/δ ligand exerts its anti-inflammatory and anti-apoptotic action, thus making the neuroprotective effect in acute ischemic stroke. In the present study, the results from the *in vivo* experiments elucidated angiogenesis as a novel mechanism underlying the protective effect of PPAR β/δ against cerebral ischemia.

PPAR β/δ is expressed in endothelial cells and has various functions in epithelial homeostasis, including cell proliferation [19-21], apoptosis [20], keratinocyte differentiation [19, 22, 23] and inflammation [23]. Piqueras et al showed that PPAR β/δ acts as a regulator of endothelial cell proliferation and angiogenesis through a VEGF-dependent mechanism [12]. In the present study, we established an *in vitro* OGD model to mimic ischemic stroke *in vivo*. Consistent with the results in normal conditions [12], activation of PPAR *β/δ by the ligand GW501516 also induced ang*iogenesis under OGD condition.

SHH is a secreted glycoprotein that regulates cell proliferation, differentiation, migration and axonal guidance [24-29] during development. SHH signaling is activated in ischemic brain injury and it participates in injury remodeling [17]. Recent studies have suggested that activation of the SHH signaling pathway could protect cortical neurons against oxidative stress, and this pathway is involved in the pathogenesis of brain ischemia and neurodegenerative disorders [30]. In the present study, we found that SHH signaling is activated in both the *in vivo* and the *in vitro* cerebral ischemia model. Furthermore, we firstly revealed that PPAR β/δ activation induced the expression of SHH protein. Subsequently, the *in vitro* experiments demonstrated that SHH knockdown attenuated

the effect of PPAR *β/δ* on cell proliferation, cell migration and tube formation of OGD-treated HUVECs, indicating that SHH mediates the effect of PPAR *β/δ* on angiogenesis.

VEGFA and Notch are important activators in angiogenesis [30]. VEGFA is the upstream regulator of Notch signaling [32, 33]. It has been suggested that activation of SHH signaling induces the expression of VEGFA as well as angiopoietin-1 and -2 [34], to promote angiogenic blood vessel growth. In this study, the results demonstrated that SHH also mediates the effect of PPAR *β/δ on* VEGFA and Notch1 expression.

In conclusion, the present study firstly demonstrated that PPAR *β/δ* activates SHH signaling in cerebral ischemia, and SHH mediates the effect of PPAR *β/δ* on angiogenesis. Our findings indicate that the regulation of PPAR β/δ/SHH/VEGF/Notch1 pathway may be a novel therapeutic intervention for cerebral ischemia.

Disclosure of conflict of interest

None.

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