

The Intrinsic PEDF is Regulated by PPAR γ in Permanent Focal Cerebral Ischemia of Rat

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Received: 20 January 2012/Revised: 17 May 2012/Accepted: 8 June 2012/Published online: 20 June 2012
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Abstract Inflammatory damage plays a pivotal role in cerebral ischemia and may represent a target for treatment. Pigment epithelium-derived factor (PEDF) is proven to possess neuroprotective property. But there is little known about the intrinsic PEDF after cerebral ischemia. This study evaluated the time course expression of the intrinsic PEDF and its underlying regulation mechanisms after cerebral ischemia. Male Sprague–Dawley rats were subjected to permanent middle cerebral artery occlusion. Telmisartan (PPAR γ agonist) and GW9662 (PPAR γ antagonist) were systemically administered to explore the effect on PPAR γ , PEDF, NF- κ B and MMP-9 expression at 24 h after cerebral ischemia by western blot and qRT-PCR. The neurological deficits, brain water content and infarct volume were measured. Compared with normal group, the expressions of PEDF and PPAR γ decreased, and the expression of NF- κ B and MMP-9 increased at early stage after ischemia ($P < 0.05$). Compared with the vehicle group, the decrease of PEDF and PPAR γ was significantly up-regulated and the increase of NF- κ B and MMP-9 was down-regulated by telmisartan at 24 h ($P < 0.05$). The neurological deficits, brain water content and infarct volume were dramatically alleviated by telmisartan

($P < 0.05$). Telmisartan's effects were reversed by GW9662 co-administration ($P < 0.05$). The expression of intrinsic PEDF was down-regulated at the early stage of cerebral ischemia. The protective effects of intrinsic PEDF by activating PPAR γ pathway may be one of the strategic targets for cerebral ischemic therapies.

Keywords Peroxisome proliferator-activated receptor-gamma · Pigment epithelium-derived factor · Matrix metalloproteinases-9 · Nuclear factor- κ B · Cerebral ischemia · Telmisartan

Introduction

Ischemic stroke is the second leading cause for human death worldwide and the main cause of acquired disability [37]. Cerebral ischemia is caused by a reduction or complete blockade of blood flow to regions of the brain. Inflammatory response has been confirmed to play a pivotal role in cerebral ischemia-induced brain injury [1, 31, 32]. It is believed that anti-inflammation and neuroprotection alleviating brain damage secondary to ischemia are the promising avenues for stroke therapy [2, 9, 12].

Pigment epithelium-derived factor (PEDF), a 50-kD secreted glycoprotein, has been well established as a neuroprotective factor since identified in 1987 [33, 35, 36]. In vitro studies, PEDF displays neuroprotective property and promotes survival of various types of nerve cells [18–20]. In vivo studies, gene transfer or N-terminal peptide of PEDF attenuates the ischemic injury in retina and brain [2–4, 24, 34]. PEDF is widely expressed in central nervous system as an intrinsic factor, especially in cerebral cortex [23]. But there is little known about the expression of the intrinsic PEDF after cerebral ischemia and its underlying regulation mechanisms.

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Recently, peroxisome proliferator-activated receptor- γ (PPAR γ), activated by agonist, has drawn more and more attention owing to alleviating the infarct volume of focal cerebral ischemia in animal models and controlling inflammation [8, 10, 21, 25]. Evidences show that PPAR γ signaling is required in the course of PEDF action in vitro and vivo studies [26–30]. However, there are limited studies available for better understanding the effect of the PPAR γ signal on regulating the intrinsic PEDF.

In the study, we evaluated the time course of the intrinsic PEDF and used telmisartan (PPAR γ agonist) and GW9662 (PPAR γ antagonist) to explore the regulation mechanisms of the intrinsic PEDF and the underlying mechanism in the permanent middle cerebral artery occlusion (pMCAO) model of rats.

Materials and Methods

Animals

Male Sprague–Dawley rats (250–280 g) were supplied by the Laboratory Animal Centre of Hebei Medical University, and were allowed free access to food and water under controlled conditions (12/12 h light/dark with humidity of $60 \pm 5\%$, $22 \pm 3^\circ\text{C}$). Animal experiments were performed according to the guidelines of the Regulations of Experimental Animal Administration promulgated by the Ministry of Science and Technology of the People's Republic of China [1988] No. 134, and approved by the Committee of Experimental Animal Administration of Hebei Medical University.

Rat Model of Permanent Focal Cerebral Ischemia

A standard model of right middle cerebral artery occlusion (MCAO) was used to make permanent focal ischemia as previously described [12–14]. Briefly, rats were anesthetized with intraperitoneal injection of 10 % chloral hydrate (350 mg/kg, IP). The right common carotid artery (MCA), external carotid artery (ECA) and internal carotid artery (ICA) were exposed through a neck incision. A monofilament suture (0.32 mm for tip diameter) was introduced from the ECA into the ICA, and advanced until resistance encountered. The inserted length of filament was 18–20 mm from the carotid bifurcation and the regional cerebral blood flow (CBF) was measured to less than 20 % baseline during anesthesia. The regional CBF of the right cortex were monitored using a blood flow monitor (moor-VMS-LDF, Moor Instruments Ltd, UK) with a fiber optic probe, adhered onto the skull surface of core area supplied by the MCA (6 mm lateral and 2 mm posterior from the bregma). Sham-operated rats underwent the same surgical

procedure without insertion of the filament. Body temperature was monitored and maintained at $36.5\text{--}37.5^\circ\text{C}$.

Groups and Drug Administration

Total 120 rats were used in the study. Part 1: Forty-two rats were randomly divided into normal, 3, 6, 24, 48 and 72 h groups after operation ($n = 7$). Part 2: Another 78 rats were randomly divided into 4 groups: sham-operated group, Vehicle group, Telmisartan group, and Telmisartan + GW9662 group. Telmisartan group received telmisartan (Boehringer Ingelheim, Ingelheim, Germany) by feeding tubes at 30 mg/kg dissolved in PBS 1 h before MCAO for avoiding emesis and then once daily thereafter. Vehicle groups received equal volume PBS in the same manner. Telmisartan + GW9662 group received telmisartan before operation and GW9662 (Sigma-Aldrich, USA) after MCAO immediately injected intraperitoneally at 4 mg/kg dissolved in 3 % DMSO diluted in PBS.

Evaluation of Neurological Deficit

Neurological deficit scores were evaluated by an examiner blinded to the experimental groups before rats were killed ($n = 6$). The deficits were scored on a modified scoring system [14] as follows: 0, no deficits; 1, difficulty in fully extending the contralateral forelimb; 2, unable to extend the contralateral forelimb; 3, mild circling to the contralateral side; 4, severe circling; and 5, falling to the contralateral side. The higher the neurological deficit score, the more severe impairment of motor motion injury.

Measurement of Brain Water Content

Brain water content was measured using the standard wet-dry method [15]. Rats were anesthetized with 10 % chloral hydrate and the brains were quickly removed ($n = 8$). A coronal brain slice (3 mm thick) was cut after dissecting free 4 mm of frontal pole. Then the coronal slice was divided into the ipsilateral and contralateral hemispheres, which were packaged with pre-weighed tin foil. The packed slices were immediately weighed on an electronic balance to obtain wet weight and then dried for 24 h in oven at 100°C to get the dry weight. Brain water content was calculated as $(\text{wet weight} - \text{dry weight})/(\text{wet weight} - \text{tin foil weight}) \times 100\%$.

Measurement of Infarct Volume

Infarct volume was determined by 2, 3, 5-triphenyltetrazolium chloride (TTC) at 24 h in the 4 group ($n = 6$). Animals were euthanized and the brains quickly collected. Brain tissue was sliced into five coronal sections (3 mm

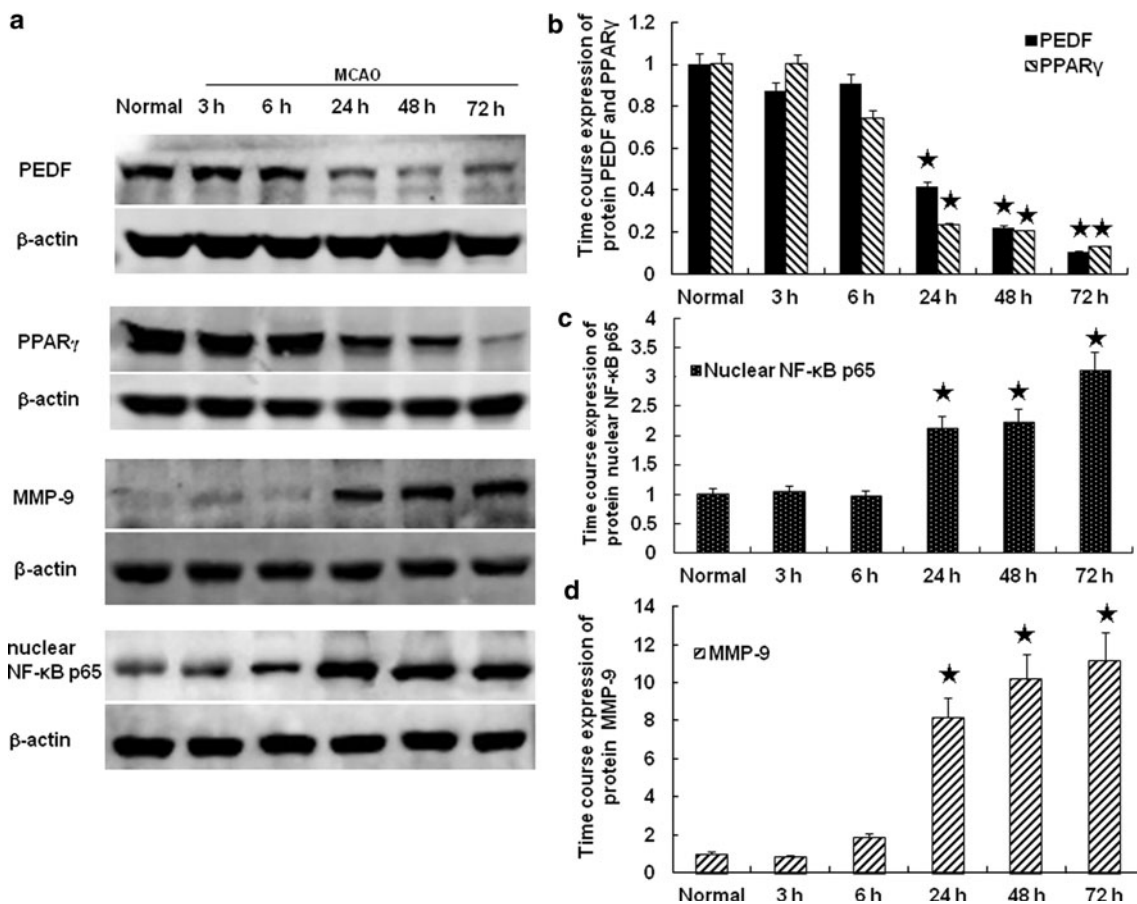


Fig. 1 The time course protein expression of PEDF, PPAR γ , NF- κ B p65 and MMP-9. Compared with normal group, protein PEDF and PPAR γ was obviously decreased from 24 to 72 h (a, b), but protein

NF- κ B p65 and MMP-9 was obviously increased from 24 to 72 h (a, c, d), * $P < 0.05$ versus normal group

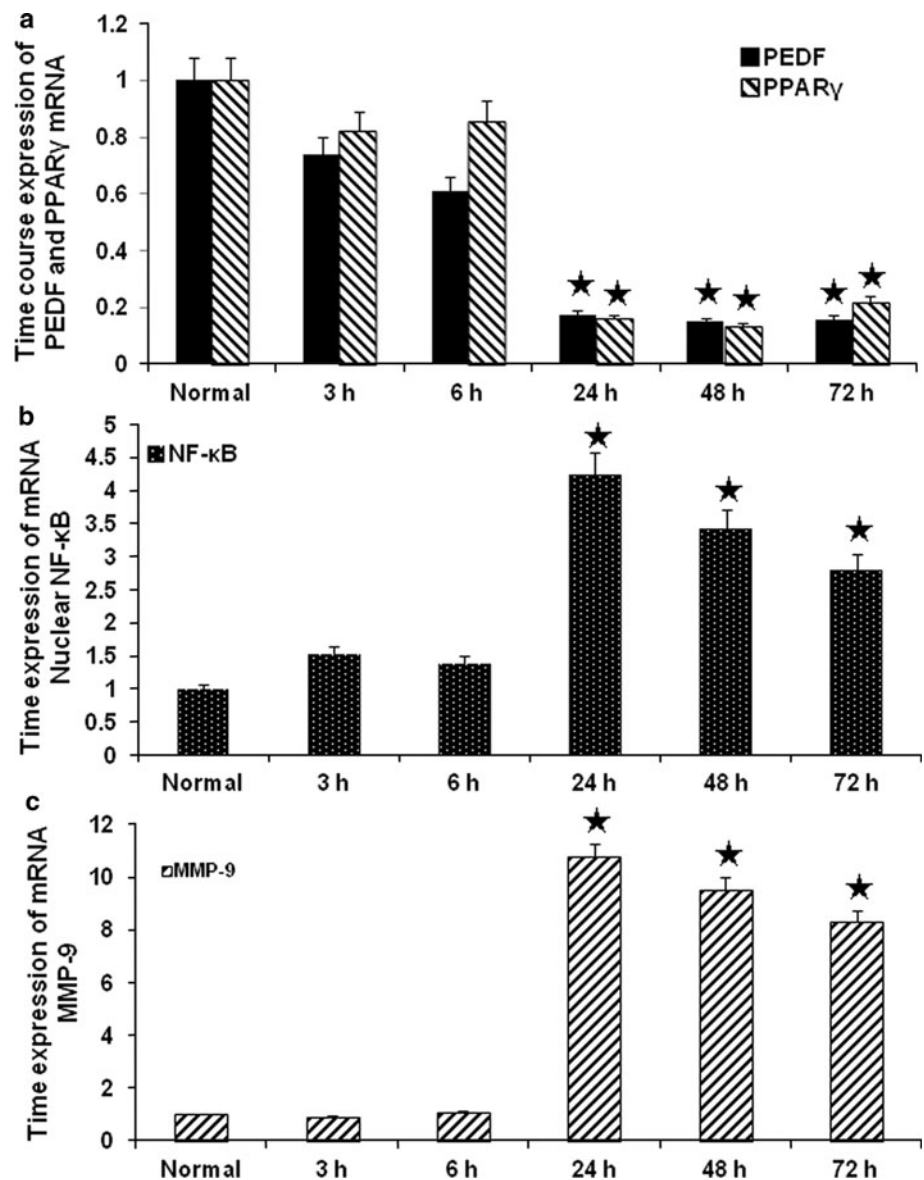
thick), stained with a 2 % solution of TTC at 37 °C for 20 min [16], followed by fixation with 4 % paraformaldehyde. Normal tissue was stained deep red, while the infarct area was stained a pale gray color. TTC-stained sections were photographed and the digital images were analyzed using image analysis software (Image-Pro Plus 5.1; Media Cybernetics, Inc, Bethesda, MD, USA) to calculate the infarct volume. To compensate for the effect of brain edema, the percentage hemisphere lesion volume was calculated by the following formula [17]: [total infarct volume – (volume of ipsilateral hemisphere – volume of contralateral hemisphere)]/contralateral hemisphere volume \times 100 %.

Western Blot

The protein extracts were performed from the ischemic cortex in ipsilateral hemisphere with Extraction Kit (Ap-lygen Technologies Inc., Beijing, China) according to the manufacturer’s protocols (n = 4). The total protein extracts are prepared for PEDF, PPAR γ and MMP-9, and nuclear

extracts were prepared for NF- κ B. Protein concentration of the supernatant was determined using a BCA Protein Assay Reagent Kit (Novagen, Madison, WI, USA) with bovine serum albumin (BSA) as the standard. Equivalent amounts (50 μ g) of protein samples were separated by sodium dodecyl sulfate–polyacrylamide gels prior (SDS/PAGE) and transferred onto PVDF membranes (Millipore Corporation, Billerica, MA, USA). After blocking for 1 h with 5 % non-fat dry milk in PBS, membranes were incubated overnight at 4 °C with polyclonal rabbit anti-PEDF and anti-PPAR γ (1:100, Santa Cruz Biotechnology), anti-NF- κ B (1:200, Santa Cruz Biotechnology), monoclonal rabbit anti-MMP-9 (1:200, Abcam Biotechnology). After 3 washes with TPBS, membranes were incubated with secondary antibody (IRDye[®] 800-conjugated goat anti-rabbit IgG, 1:3000 dilution; Rockland, Gilbertsville, PA, USA) for 1 h at room temperature. The relative density of bands was analyzed on an Odyssey infrared scanner (LI-COR Bioscience, Lincoln, NE, USA). Densitometric values were normalized with respect to β -actin immunoreactivity to correct for any loading and transfer differences among samples.

Fig. 2 The time course gene expression of PEDF, PPAR γ , NF- κ B and MMP-9. Compared with normal group, gene PEDF and PPAR γ was obviously decreased from 24 to 72 h (a), but protein NF- κ B (b) and MMP-9 (c) was obviously increased from 24 to 72 h, * $P < 0.05$ versus normal group



RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction

Quantitative real-time polymerase chain reaction (qR-PCR) was used to analyze the levels of PEDF, PPAR γ , NF- κ B and MMP-9 ($n = 3$). Rats were anesthetized and the brains were removed and frozen in liquid nitrogen. Total RNA was extracted from the ischemic cortex in ipsilateral hemisphere using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Only samples with an OD260/OD280 value >1.8 were considered appropriate for use. Reverse transcription was performed with 2 μ g total RNA using RevertAid First Strand cDNA Synthesis Kit (Fermentas International Inc, Burlington, Canada). Forward and reverse primers were 5'-CTGTGAG

AGTCCCATGATGT-3' and 5'-AGGGGCAGGAAGAA GATGATA-3' for PEDF, 5'-GAAGACATCCCGTTCAC AAGA-3' and 5'-TGATGCTTTATCCCCACAGAC-3' for PPAR γ , 5'- CAGATGATGGGAGAGAAGCAG-3' and 5'-TGTTATGATGGTGCCACTTGA-3' for MMP-9, 5'-AG-CTCCTGTCCCAGTTCTAGC-3' and 5'-ACTCCTGGGTC TGTGTTGTTG-3' for NF- κ B. Amplification and detection were carried out with the ABI 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA) using UltraSYBR Mixture (with Rox) (ConWin biotech.Co, Beijing, China) under the following conditions: 95 $^{\circ}$ C for 10 min, 40 cycles of denaturation at 95 $^{\circ}$ C for 15 s, annealing and extension at 60 $^{\circ}$ C for 60 s. Results for each sample were collected at least three times. All the threshold cycle values of target genes were

normalized relative to that of the β -actin, and relative expression ratios were calculated via the $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

Quantitative data were expressed as mean \pm SD. Statistical comparisons were conducted using one-way ANOVA followed by SNK and LSD tests for intergroup comparisons. Differences with $P < 0.05$ were considered statistically significant.

Results

The Time Course Expression of PEDF, PPAR γ , NF- κ B and MMP-9

Western blot analysis revealed that PEDF and PPAR γ protein markedly decreased, but MMP-9 and nuclear NF- κ B

p65 protein increased from 24 h to 72 h after MCAO, compared with those in normal group ($P < 0.05$). No significant differences in the expressions of these factors were observed at 3 h and 6 h after MCAO relative to normal groups ($P > 0.05$, Fig. 1a–d). Consistent with the results of western blot, decreased PEDF and PPAR γ mRNA expressions, and increased MMP-9 and NF- κ B mRNA expressions owing to MCAO exposure were also observed from 24 to 72 h with statistically significant by quantitative real-time PCR ($P < 0.05$), but not at 3 and 6 h ($P > 0.05$, Fig. 2a–c).

Telmisartan Alleviated the Neurological Deficits

Notable neurological deficits were observed on MCAO-treated animals at 24 h after MCAO. Compared with vehicle treatment, telmisartan significantly improved neurological deficits scores after MCAO ($P < 0.05$), however, which was abolished by GW9662 co-administration (Fig. 3a).

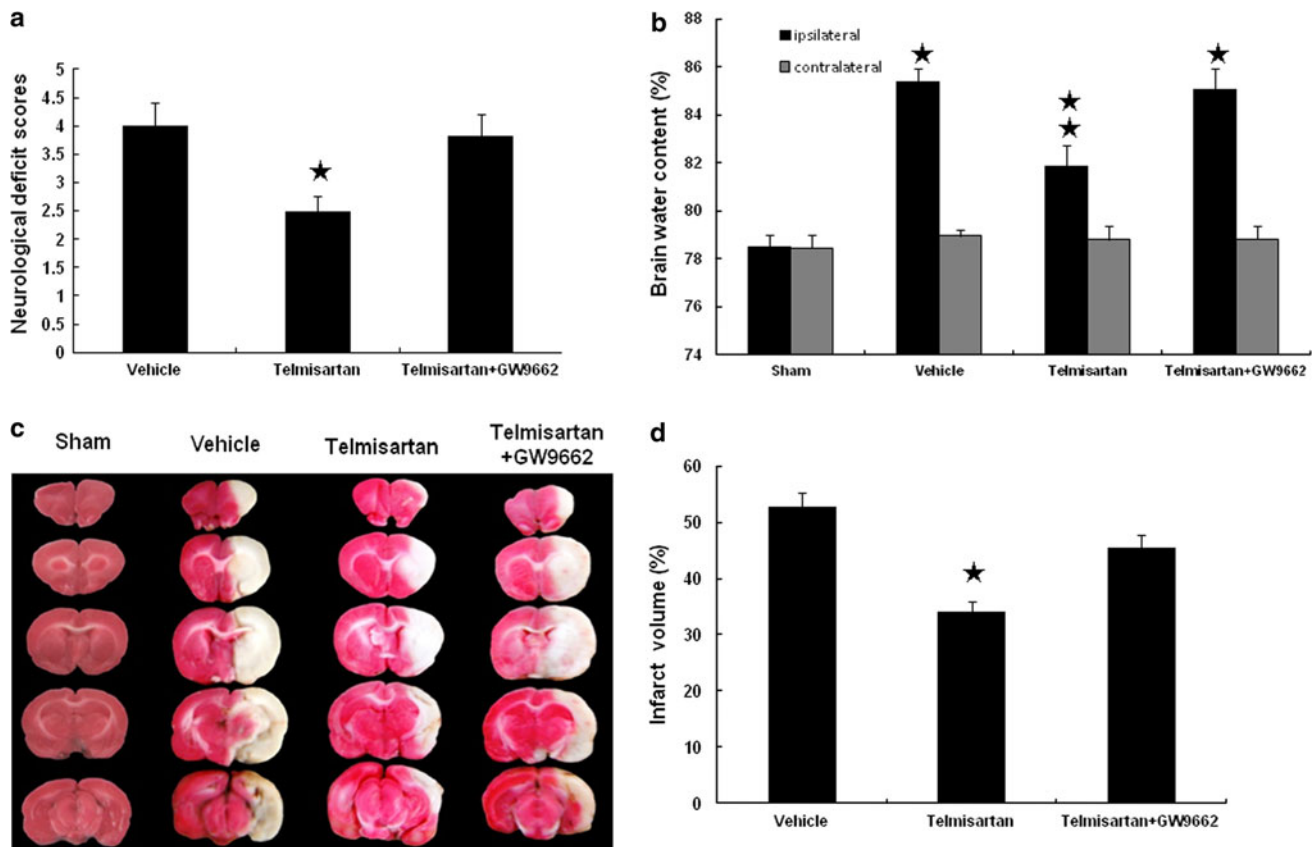


Fig. 3 The effect of telmisartan on ischemic brain. The behavioral scores were obviously improved in Telmisartan group, $\star P < 0.05$ versus Vehicle group and Telmisartan + GW9662 group. There was no significant difference between Vehicle group and Telmisartan + GW9662 group (a). The brain water content in ipsilateral hemispheres was increased in MCAO groups, $\star P < 0.05$ versus Sham group. Telmisartan significantly reduced brain water content, $\star P < 0.05$ versus Vehicle group and Telmisartan + GW9662 group.

There was no difference between Vehicle group and Telmisartan + GW9662 group in ipsilateral hemispheres. The brain water contents in contralateral hemispheres displayed no change among the four groups (b). The infarct volume was significantly alleviated in Telmisartan group, $\star P < 0.05$ versus Vehicle group and Telmisartan + GW9662 group. There was no difference between Vehicle group and Telmisartan + GW9662 group (c, d)

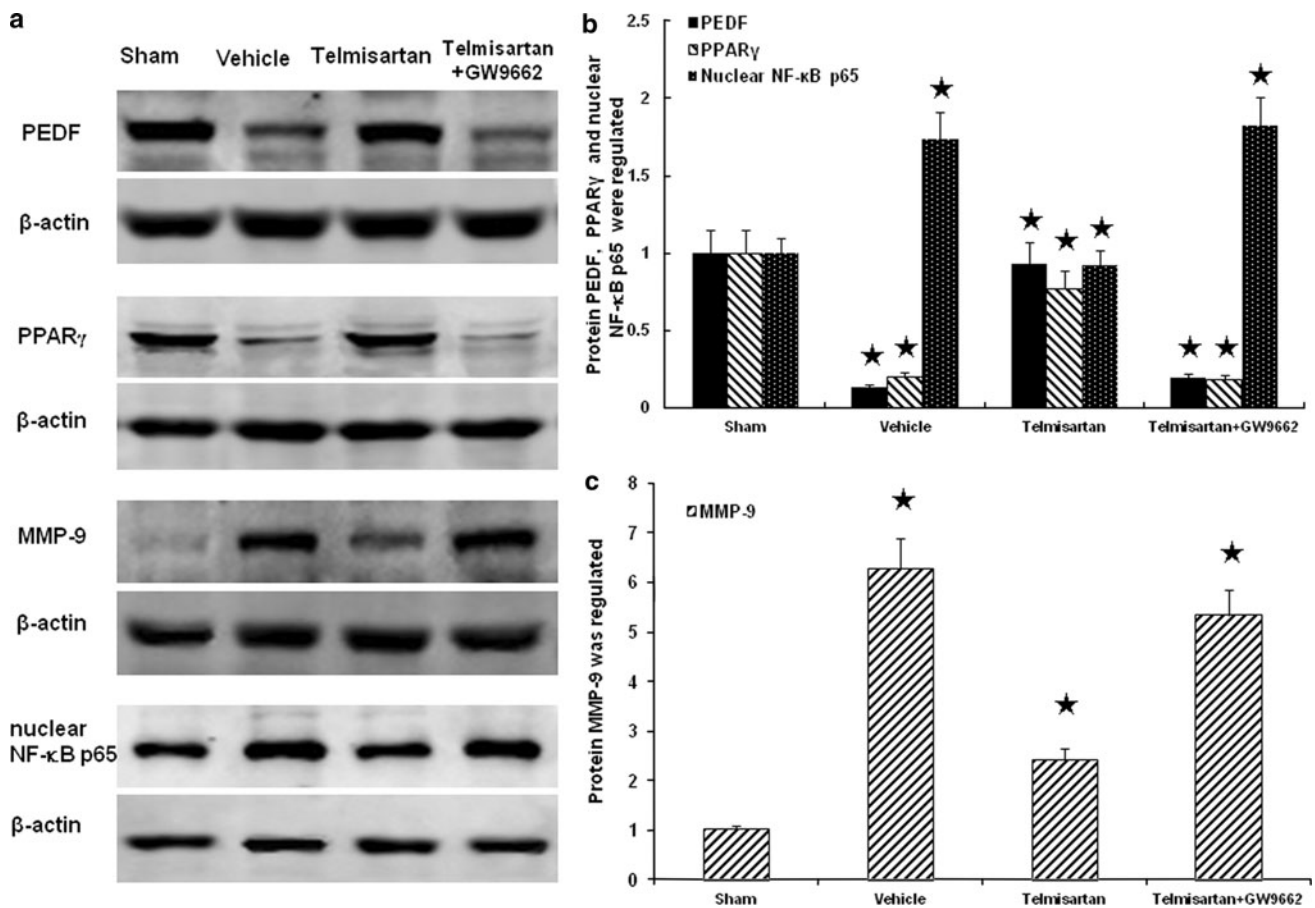


Fig. 4 The effect of telmisartan on the protein expression of PEDF, PPAR_γ, NF-κB and MMP-9. The protein expression of PEDF and PPAR_γ was obviously decreased, but the protein expression of NF-κB and MMP-9 was increased in Vehicle group and Telmisartan + GW9662 group, * $P < 0.05$ versus Sham group. In

Telmisartan group, the protein expression of PEDF and PPAR_γ was obviously increased, but the protein expression of NF-κB and MMP-9 was decreased, * $P < 0.05$ versus Vehicle group and Telmisartan + GW9662 group

Telmisartan Alleviated Brain Water Content of Ischemic Hemispheres and the Infarct Volume

At 24 h after MCAO, notably increased brain water content (%) of the ipsilateral hemispheres were detected in vehicle treatment group (85.40 ± 0.55 %), telmisartan treatment group (81.91 ± 0.86 %), and telmisartan and GW9662 co-treatment group (85.11 ± 0.81 %), compared with the sham group (78.51 ± 0.50 %, $P < 0.05$). Telmisartan treatment (81.91 ± 0.86 %) significantly alleviated brain water content, compared with vehicle treatment ($P < 0.05$). However, the alleviation of brain water content by telmisartan was abolished by GW9662 co-administration (85.11 ± 0.81 %). Brain water contents in contralateral hemispheres displayed no change among the four groups ($P > 0.05$, Fig. 3b).

The volume of cerebral infarction (% HLV) was measured by 2, 3, 5-triphenyltetrazolium chloride (TTC) staining at 24 h after MCAO and no infarct were detected in sham animals. Telmisartan administration (34.10 ± 3.78 %) significantly decreased infarct volume, compared with vehicle

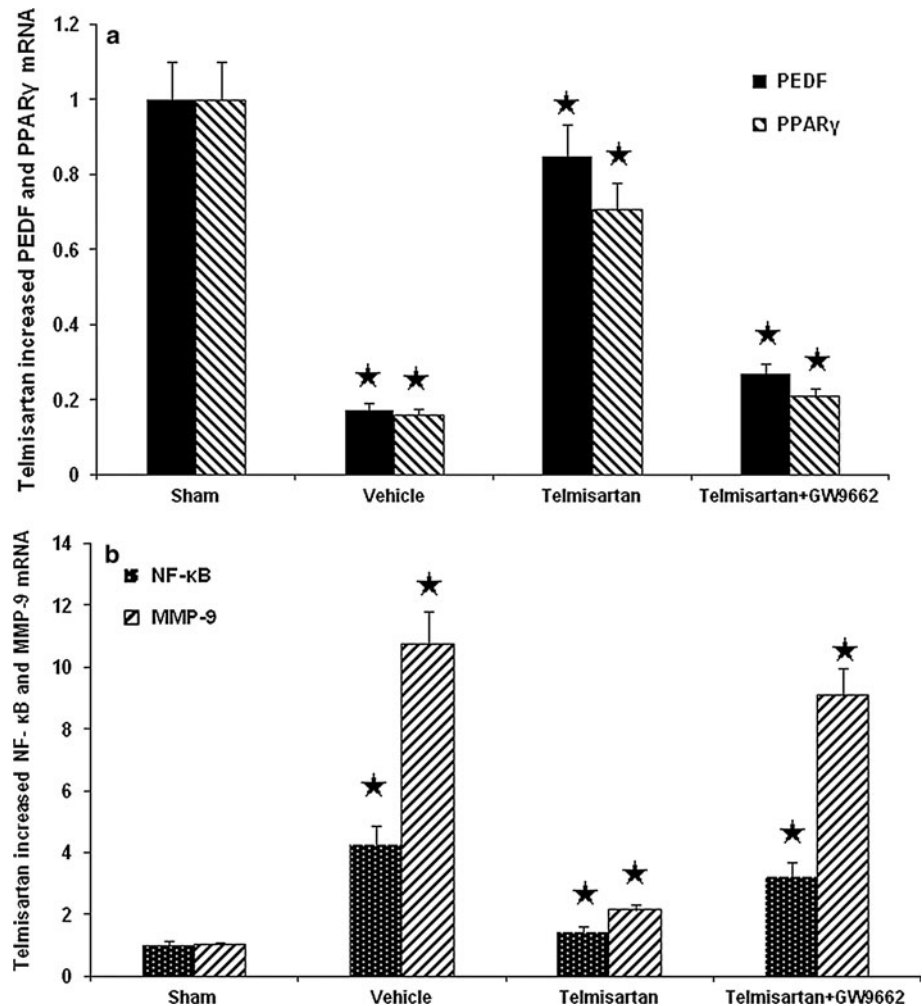
treatment (52.72 ± 7.41 %, $P < 0.05$). However, GW9662 co-administration (45.49 ± 8.12 %) reversed the alleviated infarct volume by telmisartan (Fig. 3c, d). No significant differences in infarct volume were observed between the vehicle treatment group and the telmisartan and GW9662 co-treatment group ($P > 0.05$, Fig. 3c, d).

PEDF, MMP-9 and NF-κB were Regulated by PPAR_γ After Cerebral Ischemia

PPAR_γ agonist telmisartan and antagonist GW9662 were employed to investigate the regulation of PEDF, MMP-9 and NF-κB expression at 24 h after MCAO by western blot and qR-T PCR.

Western blot revealed that the decreased protein expressions of PEDF and PPAR_γ at 24 h after cerebral ischemia were significantly up-regulated by telmisartan intervention, but the protein expressions of MMP-9 and nuclear NF-κB p65 subunit were down-regulated, relative to vehicle treatment ($P < 0.05$). The effects of telmisartan

Fig. 5 The effect of telmisartan on the mRNA of expression PEDF, PPAR γ , NF- κ B and MMP-9. The mRNA of PEDF and PPAR γ was obviously decreased, but the mRNA of NF- κ B and MMP-9 was increased in Vehicle group and Telmisartan + GW9662 group, * $P < 0.05$ versus Sham group. In Telmisartan group, the mRNA of PEDF and PPAR γ was obviously increased, but the mRNA of NF- κ B and MMP-9 was decreased, * $P < 0.05$ versus Vehicle group and Telmisartan + GW9662 group



were all reversed by GW9662 co-administration ($P < 0.05$). No significant differences in protein expression of the factors were observed between the vehicle treatment group and the telmisartan and GW9662 co-treatment group ($P > 0.05$, Fig. 4a–c).

In agreement with the results of western blot, the mRNA of PEDF, PPAR γ , MMP-9 and NF- κ B were regulated by telmisartan and GW9662 co-treatment in the same tendency by qR-T PCR ($P < 0.05$, Fig. 5a, b).

Discussion

PEDF has been well established as a neuroprotective factor exerting protection for various types of nerve cells against neurotoxic damage especially ischemic stroke [2–4, 18–20, 24, 33–36]. The over-expressed PEDF by gene transfer has been proven to reduce cerebral infarction and brain edema, alleviate inflammatory responses and degeneration of nerve cells in experimental cerebral ischemia [2]. However, there

is little known about the intrinsic PEDF's protective role and regulation mechanisms. In our study, the expression of intrinsic PEDF was significantly down-regulated at the early stage after cerebral ischemia, while the expression of PPAR γ was decreased and the expression of MMP-9 and NF- κ B was increased. The results indicated that there might be some correlations between the inflammatory factors and the intrinsic PEDF.

Several studies have demonstrated that activating PPAR γ played pivotal role in PEDF's beneficial effects [26–30]. In our study, by systemically administration of PPAR γ agonist telmisartan, the decrease of PEDF was significantly up-regulated and the increase of NF- κ B and MMP-9 was down-regulated, and the neurological deficits, brain water content and infarct volume were dramatically alleviated at 24 h after cerebral ischemia. All of the profits were reversed by PPAR γ antagonist GW9662 co-administration. The results showed that the intrinsic PEDF expression was regulated by PPAR γ at the early stage of cerebral ischemia. It's indicated that improving the intrinsic PEDF by PPAR γ -dependent pathway could provide an attractive strategy in

cerebral ischemia treatment. PPAR γ signaling played an important role in alleviating ischemic brain damage.

PPAR γ , activated by agonist such as telmisartan, is well confirmed to attenuate infarct volume and inflammatory responses after cerebral ischemia [9]. As a key transcription factor, PPAR γ inhibits inflammation factors down-stream including MMP-9 and NF- κ B [8, 10, 21, 22, 25], which were contributed to cerebral ischemia-induced inflammatory responses and brain damage [12, 31, 32]. Previous studies have proven that inflammation factors played important role in regulating the intrinsic PEDF. The expression of PEDF was reduced in the ischemic retina with inflammation factors involved in [5]. The production and release of PEDF under hypoxic conditions are reduced owing to MMP-9 over-expression [6, 7, 11]. NF- κ B inactivation is at least partially required for PEDF displaying its neuroprotection [38]. So inhibiting NF- κ B and MMP-9 could facilitate the restoration and neuroprotection of PEDF. In our study, the over-expression of NF- κ B and MMP-9 was down-regulated by PPAR γ agonist telmisartan, but reversed by PPAR γ antagonist GW9662 co-administration. The results showed that PPAR γ inhibiting NF- κ B and MMP-9 was at least partially responsible for improving the intrinsic PEDF expression.

Taking together, our findings showed the expression of intrinsic PEDF was downregulated at the early stage of cerebral ischemia. The intrinsic PEDF was upregulated by PPAR γ agonist, while the expression of NF- κ B and MMP-9 was downregulated and the neurological deficits, brain water content and infarct volume were alleviated in ischemia stroke. The protective effects of intrinsic PEDF by activating PPAR γ pathway could be one of the strategic targets for cerebral ischemic therapies.

Acknowledgments This work was funded by Hebei Province (Grant no. C2010000564 and no. 10276104D). We thank technicians Ruichun Liu and Hongran Wu for their technical assistance and Prof. Yansu Guo M.D. PhD. and Prof. Weisong Duan M.D. PhD. for providing valuable suggestions.

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