Peroxisome Proliferator–Activated Receptor β/δ Alleviates Early Brain Injury After Subarachnoid Hemorrhage in Rats

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Background and Purpose—Early brain injury is proposed to be the primary cause of the poor outcome after subarachnoid hemorrhage (SAH), which is closely related to the neural apoptosis. To date, the relationship between peroxisome proliferator–activated receptor β/δ (PPARβ/δ) and nuclear factor-κB/matrix metalloproteinase-9 (NF-κB/MMP-9) pathway, both of which are closely related to apoptotic effects, has been poorly studied in SAH. The present study was undertaken to evaluate the effects of PPARβ/δ on early brain injury and NF-κB/MMP-9 pathway after SAH in rats.

Methods—SAH model was established by injecting nonheparinized autologous arterial blood into the prechiasmatic cistern of male Sprague–Dawley rats. Adenoviruses or small interfering RNAs were injected into the right lateral cerebral ventricle to, respectively, up- or downregulate PPARβ/δ expression before SAH. All animals were assessed with a neurological score and then killed at 24 hours after SAH surgery. The indexes of brain water content, blood–brain barrier permeability, and apoptosis were used to detect brain injury. The expression of PPARβ/δ, NF-κB, and MMP-9 were measured by immunohistochemistry, gelatin zymography, and Western Blot methods, respectively. In addition, GW0742, a specific agonist of PPARβ/δ, was used to treat SAH in rats, the effects of which were evaluated by neurological scoring and Evans blue extravasation.

Results—Overexpression of PPARβ/δ by adenoviruses treatment significantly ameliorated brain injury with improvement in neurological deficits, brain edema, blood–brain barrier impairment, and neural cell apoptosis at 24 hours after SAH in rats, whereas downregulation of PPARβ/δ by small interfering RNAs administration resulted in the reverse effects of the above. The expression levels of NF-κB and MMP-9 were markedly downregulated when PPARβ/δ increased after PPARβ/δ adenovirus transfection and upregulated when PPARβ/δ decreased by PPARβ/δ small interfering RNAs treatment. Moreover, GW0742 improved neurological deficits and reduced Evans blue extravasation at 24 hours after SAH.

Conclusions—PPARβ/δ’s overexpression may attenuate early brain injury after rats’ SAH administration, which reduces neural apoptosis possibly through blocking NF-κB/MMP-9 pathway. (Stroke. 2016;47:196-205. DOI: 10.1161/STROKEAHA.115.011701.)

Key Words: apoptosis ■ early brain injury ■ MMP-9 ■ PPARβ/δ ■ subarachnoid hemorrhage

Subarachnoid hemorrhage (SAH) is a devastating disease with high mortality and disability. Traditionally, cerebral vasospasm, which develops in large cerebral arteries 3 to 7 days after SAH, was considered the most crucial cause of serious consequences after SAH. However, recent studies have shown that there is little advance in improving the outcome of SAH by prevention of vasospasm. It is reported that 12% of SAH patients die before receiving medical attention and 33% within 48 hours of SAH and 50% survivors have permanent disability.1 The primary determinant of poor outcome in SAH patients was proposed to be early brain injury (EBI).2,3 EBI was described as the immediate injury of the brain after SAH, including elevation of intracranial pressure, disruption of blood–brain barrier (BBB), neuronal cell death, brain edema, and other pathophysiologic changes.4,5 Several recent studies have indicated apoptosis might play an important role in the pathogenesis of EBI after SAH.6
Peroxisome proliferator–activated receptor β/δ (PPARβ, referred to also as PPARδ) is the maximum content of the PPARs subfamily in the central nervous system, which has been implicated in the antiapoptotic pathogenesis of numerous diseases, such as diabetes mellitus, obesity, Alzheimer’s disease, and cerebral infarction. However, there is less information about the role and mechanism of PPARβ/δ in SAH. It is reported that PPARβ/δ may play a role in brain protection by regulating nuclear factor-κB (NF-κB) or matrix metalloproteinase-9 (MMP-9) expression in cerebral ischemia, spinal cord injury, and Parkinson’s disease.

MMP-9 belongs to a family of zinc-binding proteolytic enzymes that remodel the extracellular matrix in normal. But in pathological condition, MMP-9 can especially attack type IV collagen, laminin, and fibronectin, the major components of the basal lamina. In our previous study, the expression of MMP-9 was found increased greatly with laminin reduced and apoptosis increased at 24 hours after SAH in rats, whereas laminin degradation and neuronal death could be alleviated by inhibiting MMP-9 after SAH. In addition, it is indicated that MMP-9 could be downregulated by NF-κB suppression.

However, the role of PPARβ/δ in EBI after SAH is poorly understood. Meanwhile, the modulation of PPARβ/δ on NF-κB/MMP-9 pathway in brain has not been studied. As a most probable mechanism, the role of PPARβ/δ and apoptosis in EBI after SAH were studied in rats in present work.

Materials and Methods

All animal procedures were approved by the Experimental Ethics Committee at the First Affiliated Hospital of Chongqing Medical University in Chongqing, China.

Adenoviruses and Small Interfering RNAs

Two kinds of recombinant adenoviruses were used for gene transfer: (1) replication-deficient human adenoviruses containing rat PPARβ/δ cDNA (adenovirus PPARβ/δ [Ad-PPARβ/δ]), which was used to overexpress PPARβ/δ protein; and (2) adenovirus with human GFP (adenovirus GFP [Ad-GFP]) as a control to Ad-PPARβ/δ. Ad-PPARβ/δ (6×10^10 pfu/mL) and Ad-GFP (2×10^10 pfu/mL) were produced by Genechem in Shanghai of China. Both of them were stored at −80°C until use and diluted to 1.3×10^6 pfu/mL in enhanced transfection solution (Genechem) before intracerebroventricular injection. And the volume of 10 μL was used to injection in each animal (online-only Data Supplement).

Two kinds of small interfering RNAs (siRNA) were made by GenePharma in Shanghai of China: (1) PPARβ/δ siRNA disorganizing rat PPARβ/δ mRNA (si-PPARβ/δ), to silence its transcription; and (2) scramble siRNA (si-Con), a control item to si-PPARβ/δ. Either of them was injected into lateral cerebral ventricle at a rate of 2 μL/min with 10 μL of 2 μM siRNA according to manufacturer’s instruction (online-only Data Supplement and Figure I in the online-only Data Supplement).

Intracerebroventricular Injection and SAH Model Establishment

Adenoviruses or siRNA was injected into the right lateral ventricle before SAH. After the intraperitoneal anesthesia with chloral hydrate (4 mg/kg), rat’s head was fixed in the stereotactic frame. Under sterile technique operation, the transfection site was prepared for delivery using coordinates of 1.0 mm posterior of bregma and 2.0 mm lateral of sagittal suture, at a depth of 3.5 mm. The formulated lipid was slowly injected into the lateral ventricle by a 25-gauge needle. The needle stayed in the brain for 10 minutes after injection and then the head was tilted nose-down at 30° for 30 minutes after sutured skin. Animals in control group and SAH group were injected with equal volume of saline into the lateral ventricle.

Experimental SAH was induced as reported previously with slight modifications. In brief, anesthetized animal subjects were mounted in the stereotactic frame. The needle with a rounded tip was tilted 60° in the sagittal plane and placed 8 mm anterior to bregma in the midline, with a hole into the prechiasmatic cistern facing the right side. It was lowered until the tip reached 2 mm anterior to the chiasma (±10 mm from the brain surface), and cerebrospinal fluid efflux could be observed. The amount of 0.3 mL nonheparinized fresh autologous arterial blood was slowly injected into the prechiasmatic cistern in 30 s with a syringe pump under aseptic technique. Rectal temperature was maintained at 37±0.5°C throughout the procedure, with a heating pad if required. Right after operation, 5 mL saline was injected subcutaneously to avoid dehydration. Control group animals were drilled and injected equal volume of saline. The animals were observed for 30 minutes and then returned to their cages with access to food and water. The room temperature was maintained at 26±1°C.

Experimental Protocol

Male Sprague–Dawley rats weighing 300 to 350 g were randomly divided into 2 parts containing 4 groups, respectively: Part I for PPARβ/δ overexpression: (1) control group (as a control to SAH), (2) SAH+Ad-PPARβ/δ group, and (4) SAH+Ad-PPARβ/δ silencing: (1) control group, (2) SAH group, (3) SAH+si-Con group (as a control to si-PPARβ/δ), and (4) SAH+si-PPARβ/δ group. There were 18 rats in each group. The control group and SAH group animals were injected with equal volume of saline into the lateral ventricle. In Part I, Ad-GFP or Ad-PPARβ/δ was injected into the lateral ventricle at day 0, and SAH model was produced at day 6 after the injection of adenoviruses when adenovirus had a max amplification. In Part II, siRNA injection was performed as our previous study, and SAH model was made at 24 hours after siRNA injection (day 1; Figure 1A).

All animals were deeply anesthetized and killed at 24 hours after SAH. Six rats in each group had their brains removed right after being killed, and hippocampuses were isolated, which were stored in lipid nitrogen for Western Blot and gelatin zymography experiments. Four animals in each group were perfused through the left cardiac ventricle with saline for 2 minutes followed by 150 mL of 4% paraformaldehyde in phosphate buffer solution (PBS, pH=7.4). The brains were removed and fixed in 4% paraformaldehyde for 48 hours. They were processed and embedded in paraffin. Sections of 4- to 6-μm thick were used for terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) and immunohistochemistry staining. Another 4 rats in each group were taken for brain water content detection (dry/wet method). The rest 4 rats in each group were used to detect BBB permeability (Evans blue [EB] method) in Part I and II experiments.

In addition, 12 male Sprague–Dawley rats (weighing 300–350 g) were randomly divided into 3 groups: sham-operation group, SAH+vehicle group, and SAH+GW0742 group. GW0742, a special agonist of PPARβ/δ, was given intracerebroventricularly as the above method. Animals were used to detect EB amounts after neurological scoring at 24 hours after SAH.

Neurological Scoring

Neurological deficits were evaluated at 24 hours after SAH using the modified Garcia’s method (Table I in the online-only Data Supplement) by an investigator blinded to the grouping. The evaluation consists of 6 tests that can be scored 3 to 18, including spontaneous activity (0–3), symmetry movements of 4 limbs (0–3), outstretching of forelimbs (0–3), climbing (1–3), body proprioception (1–3), and response to vibrissae touch (1–3). A lower score represents a more serious neurological deficit. The sequence of behavioral test tasks was randomized for animals.
BBB permeability was detected by EB extravasation at 24 hours after SAH. At first, animals were anesthetized at 23 hours and injected intravenously 2% EB in PBS at a dose of 2 mL/kg. And 1 hour later, they were then reanesthetized and perfused through the left cardiac ventricle with saline to clear intravascular EB dye. Then the rats were decapitated, with whole brains removed and homogenized in 2 mL PBS. Trichloroacetic acid (1 mL) was added to precipitate protein, and the samples were centrifuged at 12,000 rpm for 15 minutes. The resulting supernatant was measured for absorbance of EB at 630 nm using a spectrophotometer. The results were expressed as μg EB/g tissue.

Brain water content
Brain edema was detected using the wet/dry method as previously described. Briefly, brain samples were removed quickly from the skull, placed separately into preweighed and labeled glass vials, and weighed to obtain the wet weight. The vials were subsequently placed in an oven at 105°C for 24 hours and reweighed to obtain the dry weight. The brain water content was calculated as [(wet weight−dry weight)/wet weight]×100%.

TUNEL assay
TUNEL staining on formalin-fixed paraffin-embedded sections was used to assess neuronal apoptosis in the hippocampus after SAH. The sections at the level of hippocampuses were processed as instructed with an in situ cell death detection kit (Roche, Switzerland). The procedures were according to the protocol of the kit and our previous study. Briefly, the sections were dehydrated in xylene and treated by 1% Triton X-100 for 10 minutes. Then they were incubated in newly prepared TUNEL reaction solution at 37°C for 1 hour and peroxidase (POD) solution at 37°C for 30 minutes. After neutral gum sealing sections, TUNEL-positive cells were identified, counted, and analyzed under the light microscope (Leica, Germany) by an individual who was blind to the experimental groups. The extent of brain damage was then evaluated by apoptotic index, which was the average number of positive cells counted in 5 microscopic fields at 400× magnifications in each CA1 region of hippocampus section.

MMP Zymography
MMP-9 activity was assessed using gelatin zymography method. The method was administrated according to the instruction of MMP gelatin zymography electrophoretic analysis reagent kit (GENMED, China). The hippocampus samples were homogenized in lysis buffer, including protease inhibitors, at 50 mg/mL and then centrifuged at 12,000 rpm for 15 minutes at 4°C. The total protein concentration was determined by the bicinchoninic acid (BCA) assay (Beyotime, China). Samples were loaded and separated by 8% Tris-tricine gel with 0.1% gelatin as a substrate. After separation by electrophoresis, the gel was renatured for 1 hour and then incubated with digestive buffer at 37°C for 36 h. Then the gel was stained with 0.5% coomassie blue R-250 for 60 minutes and then destained for 6 to 24 h until clear white band appeared on blue background. MMP-9 activity reflected by the white band was quantified by Image J.

Immunohistochemistry
Immunohistochemistry staining was performed to determine the immunoreactivity of PPARβ/δ. Paraffin-embedded sections were deparaffinized and rehydrated through xylene and ethanol solutions and then antigen was retrieved in boiled water bath for 15 minutes using a citrate solution. Sections were cooled at room temperature for 60 minutes followed by a brief rinse in distilled water and washed in PBS.
for 10 minutes. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 minutes and rinsed in PBS. Nonspecific protein binding was blocked by 40 minutes in goat serum. Sections were incubated overnight at 4°C with primary antibodies directed against PPARβ/δ (diluted in 1:25, Santa Cruz). After washed in PBS, sections were incubated with goat antirabbit biotinylated antibody (ZSGB-Bio, China) for 40 minutes at room temperature. 3,3-Diaminobenzidine was used as chromogen, and counterstaining was performed with hematoxylin. Sections incubated in PBS were used as negative controls. Microscopy of the immunohistochemically stained tissue sections was performed by an experienced author blinded to the experimental condition. The number of positively immunostained cells in the CA1 region of hippocampus was counted randomly in 5 microscopic fields at 400× magnifications.

Western Blot
Frozen hippocampus samples were mechanically homogenized in radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime). Lysates were centrifuged at 12000 rpm for 15 minutes at 4°C. The protein concentration was estimated using the BCA Protein Assay Kit (Beyotime). Samples (40 μg per lane) were separated by 10% SDS-PAGE and electro-transferred onto a polyvinylidene difluoride membrane (Millipore). The membrane was blocked with 5% bovine serum albumin for 1 hour at room temperature and incubated overnight at 4°C with primary antibodies directed against PPARβ/δ (Santa Cruz), NF-κB (Cell Signaling), and MMP-9 (Abcam) diluted in WB primary antibody diluents at 1:200, 1:1000, and 1:1000, respectively. The glyceraldehyde-3-phosphate dehydrogenase (diluted in 1:1000; Abcam) was used as a loading control. After the membrane was washed for 10 minutes each for 3 times in PBS+TWEEN 20, it was incubated with the appropriate horseradish peroxidase–conjugated secondary antibody (diluted 1:3000 in PBS+TWEEN 20; Beyotime) for 1 hour. Densitometry analysis was performed with the Chemi-Doc detection system and Quantity-One software (Bio-Rad).

GW0742, A Special Agonist of PPARβ/δ
Briefly, GW0742 (Santa Cruz, 67 μg/kg) was injected into right lateral cerebral ventricle at 30 minutes before SAH operation. Dimethylsulfoxide was used in sham group and in SAH-vehicle group. At 24 hours after SAH, neurological scoring and EBI extravasation were detected in each group.

Statistical Analysis
All data were presented as mean±standard deviation. SPSS 17.0 was used only for statistical analysis of the data. The Chi-square test was used to compare mortality rates among groups. The Mann–Whitney U test was used to compare the behavior and activity scores among groups. The other measurements were analyzed by 1-way analysis of variance or Student's t test. Statistical significance was inferred at P<0.05.

Results
Mortality and Neurological Scores
We performed 80 SAH surgeries (Figure 1C), and 24 animals (30%) died because of SAH reactions within 24 hours after surgery. Mortality rates were 33.3% in the SAH groups in 2 experimental parts (8 of 24 animals), 30% in the Ad-GFP group (3 of 10 animals), 5.6% in the Ad-PPARβ/δ group (1 of 18 animals), 30% in the si-Con group (3 of 10 animals), and 50% in the si-PPARβ/δ group (9 of 18 animals). None of the animals died in control group (0 of 24 animals). There were significantly increased mortality in SAH, SAH+Ad-GFP, and SAH+si-Con groups as compared with control group (P<0.01 for all; Figure 2A). However, the overexpression of PPARβ/δ reduced the mortality in SAH+Ad-PPARβ/δ group (P<0.05 versus SAH group). Although there was no statistical difference between the SAH group and SAH+si-PPARβ/δ group (P>0.05), a worse tendency of mortality after interfering PPARβ/δ was found (Figure 2A).

Mean neurological scores of each group are compared in Figure 2B. Neurological score of rats with SAH was significantly lower than that of control group at 24 hours after SAH surgery.
There was a significant alleviation in neurological deficits at SAH+Ad-PPARβ/δ group ($P<0.01$ versus SAH group) and a deteriorative effect in SAH+si-PPARβ/δ group ($P<0.05$ versus SAH group), which indicates that PPARβ/δ was an important factor to prevent neurological injury.

**BBB Permeability**

SAH produced marked extravasation of EB dye into brain tissues at 24 hours ($P<0.001$ versus control group; Figure 2C), which explained BBB rupture after SAH operation. Also enhanced BBB permeability was found in SAH+Ad-GFP group and SAH+si-Con group as compared with control group ($P<0.001$ both). Treatment with Ad-PPARβ/δ markedly decreased the amount of EB extravasation in both hemispheres ($P<0.01$ versus SAH group). But more extravasation of EB dye in SAH+PPARβ/δ siRNA than that of SAH group accounted for a further broken BBB ($P<0.05$ versus SAH group).

**Brain Water Content**

Significant increase of water content in both hemispheres was found in rats at 24 hours after SAH administration compared with sham-operated rats ($P<0.01$, Figure 2D). Brain water content enhanced further after PPARβ/δ silenced ($P<0.05$ versus SAH group). However, it was reduced a lot by SAH operation followed by Ad-PPARβ/δ’s treatment to a parallel level as that of sham operation animals, which also reflected the cerebral protection role of PPARβ/δ ($P>0.05$ versus control group and $P<0.05$ versus SAH group).

**TUNEL Staining**

Only few of TUNEL-positive cells were observed in sham-operated rats under microscopic fields at 400× magnifications (Figure 3A). After SAH processed, apoptotic cells staining with 3,3-diaminobenzidine increased extremely in the CA1 region of hippocampus ($P<0.001$ versus control group; $P<0.001$ versus control group; $P<0.01$ vs control group; $P<0.05$ versus SAH group).

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<th>Group</th>
<th>Positive cells (x±s, n=4)</th>
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<tbody>
<tr>
<td>Control</td>
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<tr>
<td>SAH</td>
<td>26.5±6.19</td>
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<tr>
<td>SAH + Ad-PPARβ/δ</td>
<td>14.0±2.45</td>
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<tr>
<td>SAH + si-PPARβ/δ</td>
<td>37.5±6.56</td>
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Figure 3. A. Few positive cell was observed in control group. B, Subarachnoid hemorrhage (SAH) operation induced considerable apoptosis of hippocampus pyramidal cells in rats. C, There was marked reduction of TUNEL cells by adenovirus peroxisome proliferator-activated receptor β/δ (Ad-PPARβ/δ) treatment. D, Neuronal apoptosis was increasing in si-PPARβ/δ group ($P<0.001$ vs control group; $P<0.01$ vs SAH group).
Remarkable reduction of TUNEL-positive staining was counted as a result of treatment with Ad-PPARβ/δ (P<0.01 versus SAH group, Figure 3C), which demonstrated an essential relation of PPARβ/δ expression with neuronal death. Inversely, interfering PPARβ/δ expression produced a much more pyramidal cell death degree in hippocampuses compared with that of SAH group rats (P<0.01, Figure 3D).

**PPARβ/δ Expression**

Western Blot assay in both parts of experiments showed downregulation of PPARβ/δ expression after SAH operation and Ad-GFP or si-Con treatment (P<0.01 versus control group, Figure II in the online-only Data Supplement). No significant differences of its expression were found between SAH group and SAH+Ad-GFP group or SAH+si-Con group (P>0.05 both), which mean that GFP and scramble siRNA had not interfered experiments in both parts and were exactly correct control items for Ad-PPARβ/δ and si-PPARβ/δ. The upregulation of PPARβ/δ expression was found in SAH+Ad-PPARβ/δ group (P<0.01 versus SAH group and P>0.05 versus control group; Figure IIA in the online-only Data Supplement), which confirmed that the recombinant PPARβ/δ adenoviruses possessed excellent quality and successfully transfected into brain tissue. Conversely, PPARβ/δ expression was suppressed further in SAH+si-PPARβ/δ group animals compared with SAH group showing well interfering effect of PPARβ/δ siRNA (P<0.01 versus SAH group, Figure IIB in the online-only Data Supplement).

Immunohistological staining for PPARβ/δ showed that PPARβ/δ expressed mainly in the cytoplasm and some in the nucleus in CA1 pyramidal cells of rat’s hippocampus (Figure 4A). There was a great reduction of PPARβ/δ immunoreactivity after SAH operation in comparison with that of sham-operated rats (P<0.01, Figure 4B). Figure 4C demonstrated the validity of PPARβ/δ transfection and the same expression sites with control group (46.8±3.35 compared with SAH 31.6±7.89, P<0.001). In contrast, PPARβ/δ siRNA well controlled its expression as compared with SAH group (20.4±5.37 versus SAH 31.6±7.89, P<0.01; Figure 4D).

<table>
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<th>Group</th>
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<tr>
<td>Control</td>
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<tr>
<td>SAH</td>
<td>31.6 ± 7.89</td>
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<tr>
<td>SAH + Ad-PPARβ/δ</td>
<td>46.8 ± 3.35</td>
</tr>
<tr>
<td>SAH + si-PPARβ/δ</td>
<td>20.4 ± 5.37</td>
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**Figure 4.** A, Immunohistological staining of peroxisome proliferator-activated receptor β/δ (PPARβ/δ) were seated in cytoplasm mainly in control group at 400x magnifications. B, PPARβ/δ-positive cells were reduced in subarachnoid hemorrhage (SAH) group (*P<0.01 vs control group). C and D, The immunoreactivity of PPARβ/δ after Ad-PPARβ/δ and si-PPARβ/δ treatment (#P<0.001 and **P<0.01 vs SAH group).
MMP Activity
MMP-9 activity was quantified by gelatin zymography method in 2-parts experiments (Figure 5), which showed an increased expression of activated MMP-9 in the SAH group, Ad-GFP+SAH group, and si-Con + SAH group (P<0.05 all versus control group). Compared with SAH group in Part I, the intensity of the MMP-9 protein band was controlled well by PPARβ/δ’s transfection into rat’s brain (P<0.01 versus SAH; Figure 5A). The augment of MMP-9 activity, on the contrary in Part II, was found owing to PPARβ/δ siRNA’s effect (P<0.05 versus SAH group, Figure 5B), which demonstrated a negative correlation between PPARβ/δ expression and MMP-9 activity.

Expression of PPARβ/δ, NF-κB, and MMP-9 Protein in Experiment of Part I
At 24 hours after SAH operation, the expression level of PPARβ/δ was decreased a lot compared with that of sham-operated rat’s hippocampuses by Western blot method (P<0.01, Figure 6A). There was not any statistically significant difference in PPARβ/δ expression between SAH group and SAH+Ad-GFP group (P=0.263). The transfection of Ad-PPARβ/δ into brain tissue markedly increased the expression of PPARβ/δ protein in hippocampuses (P<0.01 versus SAH group and P>0.05 versus control group).

The expression levels of NF-κB and MMP-9 were negatively related to PPARβ/δ expression. Both of NF-κB and MMP-9 expression increased in SAH group rats compared with those of sham-operated rats (P<0.01 for NF-κB; P<0.001 for MMP-9). However, after Ad-PPARβ/δ treatment followed by SAH operation, the increased expression of NF-κB and MMP-9 was both significantly reduced in comparison with those of SAH group (P<0.01 both).

Expression of PPARβ/δ, NF-κB, and MMP-9 Protein in Experiment of Part II
Western blot assay has showed that with downregulation of PPARβ/δ expression in hippocampus after SAH, the expression of NF-κB and MMP-9 were increased both in Part I and II experiments. In Part II, PPARβ/δ expression was suppressed signally by PPARβ/δ siRNA treatment, which demonstrated that the siRNA successfully disrupted PPARβ/δ transcription (P<0.01 versus SAH group, Figure 6B). There was not any statistical difference between SAH group and SAH+si-Con group in the expression of PPARβ/δ and NF-κB/MMP-9 (P>0.05 for all). With PPARβ/δ expression restrained, the expression of NF-κB and MMP-9 increased much more than those of SAH group (P<0.01 for NF-κB, P<0.05 for MMP-9), showing a paralleled effect just as reported.17

Protection of GW0742 in SAH
According to the above methods, neurological deficits and BBB broken were detected after GW0742 treatment in SAH. The results showed that GW0742 enhanced the neurological

Figure 5. The region with gelatinase activity showed marked expression of active matrix metalloproteinase-9 (MMP-9; 92 kDa) in hippocampuses at 24 hours after subarachnoid hemorrhage (SAH). A, Activated MMP-9 was showed by densitometric analysis of highlight bands in Part I experiment (*P<0.001 vs control group; #P<0.01 vs SAH group). B, The variation of MMP-9 activity in Part II experiment was showed (P<0.05 vs control group; #P<0.05 vs SAH group).
scoring and reduced EB extravasation as compared with SAH+vehicle group (P<0.05 both; Figure III in the online-only Data Supplement).

**Discussion**

In the present study, the EBI was aggravated at 24 hours after SAH in rats. Higher mortality and more severe neurological deficits were observed in SAH-operated animals when BBB permeability, brain water content, and apoptotic cells increased. The above trend of mortality, neurological deficits, brain injury, and neuronal apoptosis were further aggravated by PPARβ/δ silenced, whereas NF-κB and MMP-9 expression were enhanced obviously. However, when we used PPARβ/δ adenovirus to upregulate its expression with suppression of NF-κB and MMP-9 as compared, respectively, with SAH group (#P<0.01 vs SAH group). PPARβ/δ expression was decreased more by RNA interference followed by SAH operation than SAH+si-PPARβ/δ group (#P<0.01 vs SAH group). Meanwhile, the expression of NF-κB and MMP-9 in SAH were enhanced greatly in comparison with SAH group (#P<0.01 of NF-κB and P<0.05 of MMP-9).

**Figure 6.** A, Peroxisome proliferator–activated receptor β/δ (PPARβ/δ) expression was decreased at 24 hours after subarachnoid hemorrhage (SAH; *P<0.01 vs control group) with the expression of nuclear factor-xB (NF-xB) and matrix metalloproteinase-9 (MMP-9) increased compared with control group (**P<0.01 for NF-xB and P<0.001 for MMP-9). Adenovirus (Ad)-PPARβ/δ transfection exactly increased the expression of PPARβ/δ (#P<0.01 vs SAH group and P>0.05 vs control group) and decreased the expression of NF-κB and MMP-9 as compared with SAH group (#P<0.01 vs SAH group). B, PPARβ/δ expression was decreased more by RNA interference followed by SAH operation than SAH+si-PPARβ/δ group (#P<0.01 vs SAH group). Meanwhile, the expression of NF-κB and MMP-9 were enhanced greatly in comparison with SAH group (#P<0.01 of NF-κB and P<0.05 of MMP-9).

PPARs are ligand-activated transcription factors that belong to the nuclear hormone receptor family. Three isoatypes are named by PPARα, PPARβ/δ, and PPARγ. It is reported recently that PPARγ protects against the development of aneurismal rupture. And PPARγ agonists (just as rosiglitazone and pioglitazone) are used to treat clinical diabetes mellitus and to study effects mainly in ischemic stroke for many years. Although there is more widespread distribution and content of PPARβ/δ in the central nervous system than other 2 subtypes—PPARα and PPARγ, little is known about the exact function of PPARβ/δ in central nervous system disease. Recently, Aleshin et al24,25 considered that PPARβ/δ linked to PPARα and PPARγ and regulated their expression, which demonstrated the important role of PPARβ/δ in neurodegenerative diseases. And PPARβ/δ expressions in different brain regions were proved also in our study (Figure IV in the online-only Data Supplement).

In stroke research, it was found that infarct size and ischemic damage increased after middle cerebral artery occlusion in PPARβ/δ knockout mice, which suggests that the expression of PPARβ/δ may be of interest for the prevention of cerebral damage. In our research, the improvement of brain injury after SAH was observed in SAH+Ad-PPARβ/δ group when the expression of PPARβ/δ enhanced extremely, as well as the exacerbation of EBI was detected after PPARβ/δ silencing. Moreover, the treatment of GW0742, a well-known agonist to specifically upregulate PPARβ/δ expression, showed a positive effect in SAH.

Recent research has demonstrated that the PPARβ/δ agonists possess antiapoptotic properties in vitro and may underlie their neuroprotective potential in vivo. It is a known fact that neuronal apoptosis plays a major role in brain injury associated with stroke. However, the exact apoptotic mechanism of EBI still remains obscure, which has hindered the
development of effective and specific treatment paradigms for SAH.

Apoptosis, an important element of EBI, was carried on at a peak in 24 hours after experimental SAH mostly in the neurons of the hippocampus, endothelial cells, and a lesser degree in the cerebral cortex. Neuronal apoptosis contributes to cytotoxic edema, as well as the broken BBB leads to vasogenic edema, which may be induced by endothelial cells death. Both of them usually develop brain edema, which is a major independent risk factor for death and a poor outcome after SAH. Both neuronal apoptosis and BBB rupture were observed at 24 hours after SAH in our present work, which may be the cause of increased brain water content. Further researches were in urgent need to explore a key factor in the development of apoptosis in brain research.

Fortunately, apoptosis associated intimately with MMP-9 has been involved in the pathogenesis of brain injury after ischemia and many neurodegenerative disorders. Lucivero et al found that the expression of MMP-9 significantly increased and was related to BBB disruption, vasogenic edema, and more severe stroke in patients with ischemic stroke. Apoptosis followed SAH have been proved in our previous study. MMP-9 was implicated in SAH because of degradating collagen IV, a major component of the basal lamina, and promoting neuronal death in hippocampuses after experimental SAH. In the present work, the overexpression of PPARδ resulted in reduction of neuronal apoptosis and alleviation of BBB damage, which is directly associated with downregulation of MMP-9 activity.

It is reported that the activation of NF-κB, which is a well-known transcriptional factor to direct MMP-9 expression in the effect of proapoptosis, is associated with apoptosis in cancer cells and tissue ischemia and reperfusion. Di Paola et al found that the activation of PPARδ could interfere with NF-κB signaling and reduce the degree of brain edema after brain injury. NF-κB and MMP-9 expression in hippocampuses were increased at 24 hours after SAH in the present study, which was negatively correlated with the expression of PPARβ/δ. However, they were decreased in expression by Ad-PPARβ/δ transfection treatment in Part I and upregulated further as compared with those of SAH group by PPARβ/δ silenced in Part II.

All the above findings suggest that PPARβ/δ plays an expressive antiapoptotic role in EBI after SAH through negatively regulating the expression of NF-κB/MMP-9 pathway in rats. The cerebral protective effect of PPARβ/δ makes it a promising approach of SAH treatment in the future. Therefore, the result of the present research may be a significant evidence for targeted therapy and an innovative idea for following SAH research. Further studies are required to account for the beneficial mechanisms and to test post-treatment effects of more PPARβ/δ agonists.

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Disclosures

None.

References

Peroxisome Proliferator–Activated Receptor β/δ Alleviates Early Brain Injury After Subarachnoid Hemorrhage in Rats

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