Effect of PPAR-β/δ agonist GW0742 treatment in the acute phase response and blood–brain barrier permeability following brain injury

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The systemic response to ischemic stroke is associated with the hepatic acute phase response (APR) that modulates leukocytes recruitment to the injured brain. The inappropriate recruitment of leukocytes to the brain parenchyma can result in blood–brain barrier (BBB) breakdown. Emerging data suggest that peroxisome proliferator-activated receptor beta/delta (PPAR-β/δ) activation has a potential neuroprotective role in ischemic stroke. However, mechanisms of PPAR-β/δ mediated protection in ischemic insults remain unclear. In the present study, we determined for the first time, the effects of GW0742, a PPAR-β/δ agonist on the APR following brain injury and assessed the effects on BBB permeability and tight junction integrity via claudin-5, occludin, and zona occludens-1 expression. C57/BL6 mice were exposed to 1 hour of ischemia and received 10 minutes before reperfusion either a vehicle solution or GW0742. Hepatic expression of chemokines (C-X-C motif ligand: CXCL1, CXCL2, and CXCL10), serum amyloid A-1, tumor necrosis factor alpha, interleukin-1β, and interleukin-6 was measured, and the extent of brain and hepatic neutrophil infiltration was determined. The results showed that GW0742 treatment decreased infarct volume and edema, reactant production and neutrophil recruitment to the brain and liver, which is a hallmark of the APR. GW0742 significantly reduced BBB leakage and metalloproteinase 9 expression and upregulated the expression of tight junction proteins. These findings may help to guide the experimental and clinical therapeutic use of PPAR-β/δ agonists against brain injury. (Translational Research 2017;182:27–48)

Abbreviations: APR = acute phase response; BBB = blood–brain barrier; CBF = cerebral blood flow; CCR = chemokine receptors; CNS = central nervous system; CXCL = chemokine (C-X-C motif) ligand; DAB = diaminobenzidine; EB = Evans Blue; GAPDH = glyceraldehyde 3-phosphate dehydrogenase; GRO = growth-related oncogene; IL-1β = interleukin-1β; IL-6 = interleukin-6; IL-8 = interleukin-8; Kd = dissociation constant; KO mice = knockout mice; MCAO = middle cerebral artery occlusion; MIP-2 = macrophage inflammatory protein-2; NF-kB = nuclear factor; PBS = phosphate-buffered saline; PMN = polymorphonuclear leukocytes; PPAR-β/δ = peroxisome proliferator-activated receptor beta/delta; PPAR-γ = peroxisome

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proliferator-activated receptor gamma; SAA = serum amyloid A; TJs = tight junctions; TNF-α = tumor necrosis factor; TTC = 2,3,5-triphenyltetrazolium chloride; WT = wild-type; ZO-1 = occludens-1

AT A GLANCE COMMENTARY

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Background

PPAR-β/δ-mediated protection during ischemic stroke remains unclear. The objective of the present study was to demonstrate the neuroprotective actions of GW0742, a PPAR-β/δ agonist in a murine model of focal cerebral ischemia and reperfusion.

Translational Significance

A GW0742 treatment decreased infarct volume and edema, reactant production and neutrophil recruitment to the brain and liver, which is a hallmark of the hepatic acute phase response. GW0742 significantly reduced blood–brain barrier leakage and metalloproteinase 9 expression and upregulated the expression of tight junction proteins. These findings may help to guide the experimental and clinical therapeutic use of PPAR-β/δ agonists against brain injury.

INTRODUCTION

Ischemic stroke is a relevant entity of cerebrovascular diseases that can provoke not only death but also dramatic disabilities.[1] The mechanisms underlying neural cell injury in cerebral ischemic are not yet well understood, but many studies have clearly showed that inflammatory response has been found to play an important role in the pathogenesis of cerebral ischemia.[2] Inflammatory responses involved a sequential series of processes including increased expression of endothelial adhesion molecules and chemotactic factors,[3] activation of microglia and macrophages,[4] leukocytes infiltration,[5] and long-term neutrophil recruitment[6] that could contribute to disruption of blood–brain barrier (BBB) leading to brain edema as well as neuronal damage.[7] Thus, inflammatory processes could exacerbate brain injury and worsen the neurological outcomes. It is now well established that brain damage can initiate a strong acute phase response (APR). The significance of the role of the liver in the APR following brain injury has been demonstrated.[8] Indeed, a significant component of the hepatic APR was the induction of brain injury–specific cytokine and chemokine expression. Chemokines are a family of chemotactic cytokines that attract leukocytes toward a site of injury.[9] Many studies have attempted to identify mechanistic links between the peripheral hepatic APR and the central inflammatory response. Findings indicated that focal cerebral ischemia resulted in dynamic and widespread activation of inflammatory cytokines, chemokines, and chemokine receptors in the peripheral immune system. Indeed, ischemic stroke models and peripheral IL-1 administration have been shown to increase levels of CXCL10/interferon–inducible protein-10 in unstimulated splenocytes.[10] In addition, activating its receptor CXCR3, a concomitant increase in CXCL10 binding was associated with the increased CXCR3 expression in the ischemic brain. These changes were correlated with increased leukocyte accumulation in the ischemic brain after focal stroke.[11] Cytokine-induced neutrophil chemoattractant-1 (CINC-1/CXCL1) was the major chemokine involved in neutrophil recruitment to the brain and spinal cord following an inflammatory challenge in the rat, although the other member of the family, CXCL2 (macrophage inflammatory protein-2, MIP-2α), was also elevated.[12] The expression of neutrophil-associated CXCR2 and its ligands, CXCL1 and CXCL2, were substantially upregulated in the brain after cerebral ischemia and that treatment with an antagonist of CXCR2 could markedly reduce both expression of CXCR2 and neutrophil infiltration after stroke.[13] The APR is also characterized by increased circulating levels of acute-phase proteins generated by the liver. Serum amyloid A (SAA) is an acute-phase protein secreted mainly by hepatocytes during an inflammatory response and participated in enhancing the migration of monocytes and polymorphonuclear leukocytes to inflamed tissues during an APR.[12] Proinflammatory cytokines, such as tumor necrosis factor (TNF-α), interleukin (IL)-1 and IL-6 have been implicated as important mediators of ischemia/reperfusion injury following both focal and global cerebral ischemia.[14] TNF-α and interleukin-1β (IL-1β) are pleiotropic cytokines released by many cell types on diverse stimulation. They exerted multiple biological activities including stimulation of acute phase protein secretion, induction of CXC chemokines, and

vascular permeability.[15] These actions provided a central role in leukocyte infiltration and tissue injury after cerebral ischemia. In addition, IL-6 is a key element in the APR, mediating the synthesis of several acute phase proteins (such as C-reactive protein and serum amyloid A).[16] It also increases the expression of endothelial leukocyte adhesion molecules, further promoting leukocyte accumulation.[17] Hallmarks of the acute phase of the inflammatory response are the opening of the BBB and the infiltration of neutrophils. Pharmacologically, targeting the APR in experimentally derived brain injury models has been shown to reduce hepatic and central neutrophil recruitment. Inhibition of the hepatic APR, therefore, provides an accessible peripheral target for the prevention of leukocyte recruitment to the injured brain.[18] Peroxisome proliferator-activated receptors (PPARs) belong to the nuclear receptor family of ligand-activated transcription factors have been proposed as possible neuroprotectants.[19] Three isoforms of PPARs (-α, -γ, and -β/δ) have been identified, displaying distinct physiological and pharmacologic functions. As nuclear receptors, PPARs upregulate or downregulate genes that in turn influence many of the pathophysiological pathways involved in ischemic damage, neurodegeneration, and brain repair processes.[20] PPAR-β/δ was highly expressed in diverse tissues including the vasculature and brain, but its function outside of metabolic effects was less understood in comparison with PPAR-α and PPAR-γ.[21] PPAR- β/δ has been shown to contribute to vascular remodeling, angiogenesis, and both vascular and neuronal protection. It was reported that PPAR-β/δ KO mice had a 2-fold increase in infarct size compared with wild-type (WT) mice after focal cerebral ischemia,[22] However, mechanisms of PPAR-β/δ–mediated protection during ischemic stroke remain unclear. Since common inflammatory pathways have been recently indicated as promising pathophysiological targets for both prevention and treatment in ischemic stroke,[23] we focused on the pharmacologic inhibition of CXC chemokines mainly (CXCL1, CXCL2, and CXCL10), the acute phase protein serum amyloid A-1 (SAA-1) and proinflammatory cytokines (TNF-α, IL-1β and IL-6) by GW0742, a PPAR-β/δ agonist. Disruption of the BBB is a major pathogenic mechanism of neurological dysfunction and death after ischemic stroke. The BBB is not a rigid structure but a dynamic interface, with a range of interrelated functions that result from effective tight junctions (TJs), trans-endothelial transport systems, enzymes and the regulation of leukocyte permeation. TJs consist of several types of integral transmembrane and cytoplasmic accessory proteins, of which occludin, claudin-5 and zonula occludens-1 (ZO-1) played key roles in BBB dysfunction after ischemic stroke.[24] In addition, the matrix metalloproteinase (MMP) family is a class of zinc-dependent endoproteases responsible for degradation of structural proteins in the extracellular matrix. Activation of MMPs played an important role in the pathogenesis of various neurological diseases, including stroke. Importantly, by employing PPARδ transcriptional activity analysis and a ChIP assay, PPARδ might negatively regulate MMP-9 activity via a transcriptional mechanism.[25] On this basis, therapeutic targeting that inhibits MMP-9 activity may be a promising approach to minimizing secondary brain injury in acute stroke. Thus, protecting the BBB may be a promising strategy for developing new clinical therapies for ischemic stroke. The aim of our study was also to investigate the effect of GW0742 on BBB disruption induced by ischemic stroke and explore the underlying mechanism.

MATERIALS AND METHODS

Animals and PPAR-β/δ agonist GW0742 administration. All procedures and assessments were approved by the Animal Ethics Committee of the Faculty of medicine. These experiments were carried out in accordance with the National Institutes of Health Guide for the Care and use of Laboratory Animals (National Institutes of Health Publication No 80-23, revised in 1996). All efforts were made to minimize the number of animals used and their suffering. Male C57 BL/6J mice (body weight 25–30g, 3- to 4-month-old) (Jackson Laboratory, Bar Harbor, Maine) were used. All mice were allowed free access to food and water ad libitum before surgery under optimal conditions (temperature-controlled [22°C] room with a 12-hour light/dark cycle) for 4 weeks. A dose of 30 mg/kg/d of GW0742 has been reported to exert anti-inflammatory effects.[26,27] In addition, in previous experiments, other doses of PPAR-β/δ agonist GW0742 (0.3 mg/kg and 3 mg/kg) were also tested in a pre-treatment paradigm to most efficiently identify the optimal doses for neuroprotection, but no experiments examining neuroprotection time windows utilized GW0742 treatment after middle cerebral artery occlusion (MCAO). So, we tested the hypothesis that timing of drug treatment relative to reperfusion is an important determinate of GW0742 efficacy by treating mice 10 minutes before reperfusion with a vehicle solution (1.0% w/v. carboxymethylcellulose medium viscosity, Sigma Diagnostics, St. Louis, Mo) or PPAR-β/δ agonist GW0742 intraperitoneally (i.p.) in 3 doses (0.3 mg/

kg, 3 mg/kg, and 30 mg/kg). The mice were randomly divided into Vehicle Sham Group, Vehicle MCAO group, and 3 GW0742-MCAO groups. The sham group was only subjected to surgical procedures, whereas other animals were subjected to focal ischemia by MCAO using intraluminal thread and then reperfusion allowed by retracting the thread. The physiological variables in mice were measured at baseline, during MCAO and at reperfusion. The right femoral artery was cannulated for continuous monitoring of blood pressure and arterial blood sampling. A rectal probe was inserted to monitor core temperature. Arterial blood gases and plasma glucose were measured at baseline, before, during, and after ischemia.

Focal cerebral ischemia. Focal cerebral ischemia was induced by intraluminal MCAO using a nylon monofilament technique according to a method previously described.[28] Mice were anesthetized with chloral hydrate at the dose of 300 mg/kg, intraperitoneally. Ischemia was produced by advancing a tip rounded 30-mm nylon suture into the internal carotid artery through the external carotid artery. The animals underwent left MCA occlusion for 60 minutes and then reperfusion for was produced by withdrawal of the intraluminal nylon suture. Sham-operated mice were subjected to similar operations to expose the carotid arteries without occlusion of the middle cerebral artery. Cerebral blood flow (CBF) was continuously measured during MCAO and reperfusion using laser Doppler flowmetry (Perimed Periflux 5010 Laser Doppler System), as previously described.[29] Briefly, after initial anesthesia but before midline incision for placement of the monofilament, a skin incision was made, and the microtip of the laser-Doppler fiber-optic probe was affixed over a thinned area of skull posterior to the coronal suture and lateral to the sagittal suture over the MCA perfusion domain. The probe was left in place, and CBF was recorded during the experiment. Laser Doppler was used to measure changes in CBF from baseline, including both the decrease in CBF during occlusion and the extent of reperfusion after suture removal. Interruption of regional CBF in the middle cerebral artery territory was confirmed by documenting a $>80\%$ decrease in relative regional CBF. A return to $>50\%$ of baseline regional CBF within 5 minutes of suture withdrawal confirmed a reperfusion of the middle cerebral artery territory. Animals were exposed to 1 hour of ischemia. After 6, 24, and 48 hours of reperfusion, 12 mice in each group were sacrificed. Six of which were used to measure infarct volume, BBB permeability, and brain edema, and six of which were used for polymerase chain reaction (PCR), Western blotting, immunohistochemistry, and gelatin zymography. Fresh frozen tissue was collected and each mice brain was isolated.

Pharmacokinetic study in mice. Measurement of the concentration of GW0742 in plasma, brain, and liver was performed in mice following i.p. administration of a 0.3, 3, or 30 mg/kg dose. At 0.5, 1, 3, and 6 hours after dosing, blood was collected from the ventral aorta. After removal of the blood from the brain vessel performed by carotid brain perfusion with saline, the brain was collected. Liver was also collected, and homogenate samples were prepared. Six animals were used for each time point. A 200-μL plasma sample or a 500 μL of brain and liver samples were homogenized with 5.5 mL of saline and further diluted/homogenized in 100 μL of methanol and 5 mL of diethyl ether solution, as previously described.[30] Each supernatant was subsequently evaporated under a gentle stream of nitrogen, and the dried residue was reconstituted in 100 μL of methanol. A 20-μL volume of reconstitute was injected into a column (Cadenza CD-C18, 2 $\times$ 50 mm) and detected using liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS).

Neurological deficit evaluation. After 6, 24, or 48 hours of MCAO, neurological deficit was scored according to the 5-point scoring system. The scoring system was used as follows: (1) a score of 0: normal walk or no neurologic deficit; (2) a score of 1: failure to extend opposite forepaw fully or a mild focal neurologic deficit; (3) a score of 2: circling to the contralateral side or a moderate focal neurologic deficit; (4) a score of 3: falling to the contralateral side or a severe focal neurologic deficit; and (5) a score of 4: no spontaneous walking with depressed consciousness level. The neurological evaluation was performed by an investigator who was blinded to the study protocol.

Measurement of infarct volume, and brain edema. For infarct volume measurements, the brains were quickly removed and frozen at $-20°C$ for 15 minutes. Coronal sections of 1-mm thickness were immediately stained with 2% 2, 3, 5-triphenyltetrazolium chloride (TTC) at 37°C for 20 minutes followed by fixation with 4% paraformaldehyde. The unstained area of the brain was defined as the infarct zone and analyzed by the image analyzing system (Adobe Systems Incorporated, San Jose, Calif). No infarct was detectable in the sham animals. The sham experiments would be expected to induce an APR in the liver. Brain water content was measured by the standard

wet–dry method. After 6, 24, or 48 hours, mice were decapitated and brains were immediately removed. The two hemispheres were weighed (wet weight), separately. Then the tissues were dried at 100°C for 24 h to determine the dry weight. The degree of brain edema was calculated by the following equation: water content = (wet weight − dry weight)/wet weight × 100%.

Blood–brain barrier permeability measurement. BBB permeability was evaluated by Evans blue extravasation. Mice (n = 6 for each group) were injected with 100 μl of 4% Evans Blue (EB) (Sigma–Aldrich). Animals were perfused with phosphate-buffered saline (PBS), and the brains were removed and separated into hemispheres ipsilateral and contralateral to the MCAO. Each hemisphere was then weighed and homogenized in N, N-dimethylformamide (Sigma–Aldrich) and centrifuged for 45 minutes at 25,000 rcf. The supernatants were collected, and quantitation of EB extravasation was performed as described.[31] The supernatant was measured for absorbance at 620 nm by spectrophotometry. The extravasation was expressed as μg of Evans blue per g of wet tissue weight.

Western blotting of zonula occludens-1 (ZO-1), Claudin-5, and occludin protein expression. Western blotting (n = 6 for each group) was performed as described previously.[32] Proteins were extracted from the cortex of the ischemic side in the operated mice, and the corresponding areas of sham-operated mice were used for Western blot analysis. Membranes were incubated overnight at 4°C with the appropriate primary antibodies: rabbit polyclonal anti-occludin (#71–1500; 1:1000; Invitrogen Life Technologies) and anti-ZO-1 (#61–7300; 1:1000 Invitrogen Life Technologies) and mouse monoclonal anti-claudin-5 (#4C3C2; 1:500; Invitrogen Life Technologies) and anti-glyceraldehyde 3-phosphate dehydrogenase (anti-GAPDH, #AM4300; 1: 5000; Life Technologies) as internal reference. After washing, the membrane was incubated with horseradish-peroxidase-conjugated secondary antibody for 1.5 hours. Enhanced chemiluminescence Advance Western blotting detection reagents were used to perform immunodetection. Each sample was normalized against the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) level in the sample.

Western blotting of MMP-9 protein expression. Tissue from the infarct cerebral hemisphere was applied with cell lysate to extract the total protein (i.e., the ABC protein concentration method). Then, a 30-μg protein sample was separated by 12% sodium dodecyl sulfate polyacrylamide gel electropheresis (SDS-PAGE) then transferred to a polyvinylidene difluoride (PVDF) membrane. The PVDF membrane was blocked in 5% skim milk for 2 hours and probed with the relevant primary antibody (1:200) for 1 hour, cultured in 5% CO2 incubator at 4°C overnight, then incubated with a secondary antibody for 2 hours. The Western blot fluorescence detection kit revealed a brownish red stripe for the protein of interest, and the gel image analysis system showed the density by photograph and scan. The internal reference was GAPDH.

Western blot analysis of PPAR-β/δ protein expression. Western blotting was used to measure protein expression of PPAR-β/δ. Proteins from the brain were extracted by adding 0.5 mL of tissue lysate solution and then by centrifuging at 12,000 × *g* for 5 minutes at 4°C. The protein concentration was detected by the bicinchoninic acid assay method according to the manufacturer's directions of the protein detection kit. The proteins (50 μg per sample) were separated by 10% (weight/volume) sodium dodecyl sulfate polyacrylamide gel electropheresis (SDS-PAGE) and then transferred to a PVDF membrane. The membrane was blocked for 1 hour in PBS containing 5% fat-free milk (weight/volume) and 0.2% Tween 20 (vol/vol). The blot was incubated overnight at 4°C with antibodies either to PPAR-β/δ or GAPDH at 1: 1000 dilution, followed by incubation for 1 hour at 37°C with a secondary antibody (1: 1000). Immunoreactive bands of PPAR-β/δ and GAPDH were visualized with an enhanced chemiluminescence detection kit, and the optical density bands were detected using a gel imaging and analysis system (Bio-Rad). The protein level of PPAR-β/δ was calculated as ratios of the corresponding GAPDH protein level.

Enzyme-linked immunosorbent assay. After 6, 24, or 48 hours of MCAO, mice were sacrificed, and the liver tissue homogenates were obtained. Protein levels of CXCL1, CXCL2, CXCL10, SAA-1, IL-1β, IL-6 and TNF-α were quantified using a colorimetric enzyme-linked immunosorbent assay (ELISA, R&D Systems, Minneapolis, Minn), following manufacturer's instructions. Absorbance at 450 nm was recorded, and the concentration of the target protein was read according to the standard curve. Result was expressed as pg/mg protein.

Tissue processing. Six mice per group at each time point of 6, 24, and 48 hours after the procedure were anesthetized and perfused transcardially with 0.9% saline. Hepatic tissue samples for PCR analysis were dissected and stored at −80°C. Moreover, animals were killed and were perfusion fixed with 4% paraformaldehyde. Samples of the liver and the brain were cryoprotected in 20% and 30% sucrose solutions in PBS at 4°C before being cut in sections.

Table I. Primers and probes for the mouse chemokines CXCL1, CXCL2 (MIP-2), CXCL10 (IP-10), serum amyloid A-1 protein (SAA-1), IL-1β, IL-6, and TNF-α

Marker and probe	Forward primer	Reverse primer
CXCL1	AGCCACACTCCAACACAGC	CAGCGCTGCACAGAGAAG
CXCL2	CGCTGTCAATGCCTGAAGAC	ACACTCAAGCTCTGGATGTTCTTG
CXCL10	GCTGCCGTCATTTTCTGC	TCTCACTGGCCCGTCATC
SAA-1	CCAGGATGAAGCTACTCACCA	TAGGCTCGCCACATGTCC
IL-1β	CACCTCTCA-AGCAGAGCACAG	GGGTTCCAT-GGTGAAGTCAAC
IL-6	AGTTGCCTTCTTGGGACTGA	TCCACGATTTCCCAGAGAAC
TNF-α	AAATGGGCTCCCTCTCATCAGTTC	TCTGCTTGGTGGTTTGCTACGAC

Abbreviations: IL-1β, Interleukin-1β; *IL-6*, interleukin-6; *SAA1*, serum amyloid A-1; *TNF-α*, tumor necrosis factor.

Immunohistochemistry staining. The liver samples were cut in 10-μm-thick sections from tissue blocks. Three sections were taken per mouse. Antigen retrieval was undertaken by microwaving (650 W) the slides in 0.01-M citrate acid buffer (pH 6) before washing them in a solution of PBS and 0.05% Tween 20 (PBS-T). Endogenous peroxidases were inhibited by incubating sections for 20 minutes in 3% H_2O_2 and 30% methanol in PBS, pH 7.4, and nonspecific antigen binding sites were blocked with 10% normal goat serum. Avidin was added in a 1:50 ratio to eliminate endogenous hepatic biotin, with excess avidin subsequently removed in a PBS-T wash. All solutions of antibodies were diluted using PBS. Sections from mouse specimens were immunostained overnight with biotin and a 1:1000 dilution of the rabbit anti-mouse antibody MBS-2 (a neutrophil-specific in-house antibody), which was used as the primary antibody and omitted from control slides. After a PBS-T wash, A 1:1000 biotinylated goat anti-rabbit IgG antibody was added as a secondary antibody to label bound MBS-2. After extensive washes, the substrate of the enzyme complex, diaminobenzidine, was added with 125 μl of 30% hydrogen peroxide and 250 ml of 0.1-M phosphate buffer to visualize the peroxide labeling. Mayer's hematoxylin solution was the counterstain which aided neutrophil identification. The color changed to brown at the sites of localized reaction. For the brain tissue, 3 sections were also cut. To aid neutrophil identification, 0.05% cresyl violet was the counterstain and an anti-polymorphonuclear antibody (anti- polymorphonuclear leukocytes) was the primary antibody. For quantification of neutrophils in each tissue section, 3 representative fields were chosen, and the average number of neutrophils was calculated and was expressed as the number of neutrophils/mm^2.

RNA extraction and reverse transcription. Livers were analyzed for histology and gene expression. Total RNA from livers was extracted using the Qiagen RNeasy Mini Kit (Qiagen Ltd, Crawley, UK) and complementary DNA then synthesized using a Taqman Reverse Transcription reagent kit (Applied Biosystems, Warrington, UK), both according to manufacturer's instructions.[33] Specific primers and probes (Table I) were used to determine the messenger RNA (mRNA) expression of neutrophil chemoattractants CXCL1, CXCL2 and CXCL10, serum amyloid A-1 (SAA-1), IL-1β, IL-6, and TNF-α. The housekeeping gene GAPDH was used to normalize the input RNA for each reaction. The relative mRNA expression levels were calculated using the $\Delta\Delta$Ct method.[34]

Reverse transcriptase-polymerase chain (RT-PCR) analysis of PPAR-β/δ mRNA. Six mice per group at each time point of 6, 24, and 48 hours after MCAO were sacrificed. Brains were removed for analysis of mRNA expression by RT-PCR. Total RNA was extracted from Brains using TRIZOLl LS Reagent (Invitrogen, Carlsbad, Calif) following the manufacturer's instructions. Sequences of the primers used for RT-PCR amplification and lengths of the products (in brackets) were as follows: PPAR-β/δ (212 bp) forward 5′-GCCGCCCTACAACGAGATCA-3′ and reverse 5′-CCACCAGCAGTCCGTCTTTGT-3′. Two-step RT-PCR was carried out according to the system manual. The total reaction volume for RT was 20 μL, including 4 μL 5 × RT buffer, 2 μL of 10-mM dNTP, 1 μL of 10-U/μL RNase inhibitor, 1 μL of 10- pmol/μL oligo (dT), 1 μg of RNA template, 1 μL of ReverTra Ace-α, and 10 μL of RNase-free H_2O. The RT conditions were 30°C for 10 minutes, 42°C for 20 minutes, 99°C for 5 minutes, and 4°C for 5 minutes. The total reaction volume for PCR was 25 μL, including 5.0 μL of complementary DNA, 0.5 μL of 10-mM dNTP mixture, 2.0 μL of 25-mM $MgCl_2$, 2.5-μL of 10 × PCR buffer, 0.5 μL of forward/reverse primers, 0.125 μL of 2.5- U/μL Taq DNA Polymerase and 13.875 μL of sterile double-distilled water. The PCR conditions were 94°C for 4 minutes, 35 cycles of 94°C for 15 seconds, 57°C for 15 seconds, and 72°C for 40 seconds, followed by extension at 72.0°C for 5 minutes. The PCR products

Table II. Physiological parameters

Time interval	T (°C)	MAP (kPa)	PaCO2 (kPa)	PaO2 (kPa)	pH	Glucose (mM)
	Arterial blood gases					
Preischemia	37.3 ± 0.26	14.4 ± 1.6	5.7 ± 0.8	31.2 ± 4.9	7.34 ± 0.08	4.56 ± 0.46
Intraischemia	37.6 ± 0.25	14.5 ± 2.2	5.4 ± 0.7	31.3 ± 4.4	7.35 ± 0.07	4.49 ± 0.65
Reperfusion	37.4 ± 0.28	14.4 ± 2.1	5.6 ± 1.3	30.8 ± 5.3	7.36 ± 0.09	4.47 ± 0.74

Abbreviations: MAP, Mean arterial pressure; *PaCO2*, arterial carbon dioxide partial pressure; *PaO2*, arterial oxygen partial pressure; *T*, rectal temperature.
Variables are presented as means ± standard error of the mean. All values were within normal range, and there were no significant differences among groups (n = 6 each).

Table III. Liver and brain concentrations of GW0742 in mice were determined at 0.5, 1, 3, and 6 hours after an intraperitoneal doses (0.3, 3, or 30 mg/kg)

GW0742 dose	Time, h	Brain concentration μg/g tissue	Liver concentration μg/g tissue	Plasma concentration μg/ml	Kp (brain/plasma)	Kp (liver/plasma)
0.3 mg/kg	0.5	ND	ND	1.3 ± 0.24	ND	ND
	1	ND	ND	1.6 ± 0.37	ND	ND
	3	ND	ND	1.56 ± 0.07	ND	ND
	6	ND	ND	1.45 ± 0.02	ND	ND
3 mg/kg	0.5	3.27 ± 0.7	3.80 ± 0.89	09.16 ± 1.17	0.36	0.41
	1	3.33 ± 0.1	3.9 ± 0.36	10.3 ± 0.09	0.33	0.37
	3	3.09 ± 0.2	3.2 ± 0.42	7.68 ± 1.45	0.40	0.41
	6	4.1 ± 0.98	3.9 ± 0.78	1.54 ± 0.66	2.66	2.53
30 mg/kg	0.5	5.82 ± 0.007	5.66 ± 0.65	10.97 ± 0.53	0.53	0.51
	1	6.3 ± 0.003	6.1 ± 0.84	21.7 ± 0.38	0.29	0.28
	3	6.7 ± 0.003	6.13 ± 0.65	7.23 ± 0.87	0.92	0.84
	6	6.98 ± 0.004	6.75± 0.74	2.36 ± 0.78	2.95	2.86

Abbreviations: Kp, brain/plasma or liver/plasma ratio; *ND*, no data.
Data are presented as mean ± standard error of the mean values (n = 6 at each time point).

were visualized after electrophoresis on a 1% low melt point agarose gel and stained with 0.5 μg/mL ethidium bromide for 10 minutes. The integrated gray values of the product bands were measured using a gel imaging and analysis system (Bio-Rad, Hercules, Calif). The GAPDH served as an internal control, to the level of which the amount of target mRNA was normalized.

RT-PCR detection of MMP-9 mRNA expression. Six mice in each group were decapitated. The ischemic hemispheres were randomized into 2 parts: one for extracting total RNA according to the Trizol kit instructions and another for Western blot and gelatin zymography. In a 25-μl reaction volume, PCR was performed as follows: initial denaturation for 4 minutes at 94°C, followed by denaturation for 30 seconds at 94°C, annealing for 30 seconds at 62°C and extension for 2 minutes at 72°C for 35 cycles and finally extension for 10 minutes at 72°C. Electrophoresis was used to separate the PCR-amplified products, and the results were imaged and analyzed by a gel image analysis system. Messenger RNA levels were normalized to the endogenous control, GAPDH expression.

Gelatin zymography assay for MMP-9 activity. MMP-9 activity was assessed by gelatin zymography assay. Tissue from the infarct cerebral hemisphere was applied with cell lysate to extract the total protein (i.e., the ABC protein concentration method). Then, a 30-μg protein sample was separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with 1% gelatin. After electrophoresis, SDS was eluted by incubating the gel in eluate containing 2.5% Triton X-100 for 20 minutes thrice, and Triton X-100 was then washed out by motion. The gel was incubated in substrate buffer for 48 hours at 37°C. After incubation, the gel was stained with 0.5% Coomassie Brilliant Blue solution for 4 hours. The gel was placed into a bleaching gel solution to destain until the digested band appeared, and then the gel was photographed for analysis.

Table IV. Determination of GW0742 fractional occupancy

GW0742 dose	Time, h	Brain free unbound concentration (Cu,brain)	Kd (μM)		Cu,brain/Kd		EC50 (μM)	
			Kd $_{PPAR-\gamma}$	Kd $_{PPAR-\beta/\delta}$	PPAR-γ	PPAR-β/δ	PPAR-γ	PPAR-β/δ
3 mg/kg	0.5	1.75 ± 0.2	2.42 ± 0.37	0.80 ± 0.08	0.72	2.18	2.4 ± 1.3	0.002 ± 0.001
	1	1.76 ± 0.1			0.73	2.2		
	3	1.87 ± 0.3			0.77	2.33		
	6	1.97 ± 0.1			0.81	2.46		
30 mg/kg	0.5	3.55 ± 0.4			1.46	4.43		
	1	4.25 ± 0.1			1.75	5.31		
	3	4.33 ± 0.3			1.78	5.41		
	6	4.53 ± 0.1			1.87	5.66		

Abbreviations: Cu,brain, Brain free unbound concentration; *Kd*, dissociation constant.
Data are presented as mean ± standard error of the mean.

Statistical analysis. Data are expressed as mean ± standard error of the mean (SEM). An unpaired *t* test was used for comparisons between 2 groups. Reperfusion CBF was determined from laser Doppler units as a percent change from baseline CBF. For comparisons between multiple groups, the significance of differences in between-group means was determined by 2-way analysis of variance combined with a post hoc test. Dissociation constant (Kd) was calculated by nonlinear regression. A P value < 0.05 was considered to be statistically significant. Associations between inflammatory markers and clinical outcomes were analyzed by Pearson's correlation coefficient. GraphPad Prism version 6 (GraphPad Software Inc, La Jolla, Calif) was used to perform all statistical analyses.

RESULTS

Physiological parameters. There were no significant differences for these variables (mean arterial blood pressure, arterial carbon dioxide partial pressure, arterial oxygen partial pressure, pH, rectal temperature, and blood glucose) at baseline, during MCAO and at reperfusion. These variables remained in the normal range during the experimental period observed (Table II).

Quantification of GW0742 levels. The plasma, liver, and brain concentrations of GW0742 in mice were determined at 0.5, 1, 3, and 6 hours following i.p. administration of 0.3, 3, and 30 mg/kg. Mean plasma concentrations of Cmax in mice treated with 0.3, 3, and 30 mg/kg dose were 1.6, 10.3, and 21.7 μg/ml, respectively, at 1 hour, and the plasma concentrations decreased 6 hours after dosing for 3 mg/kg and 30 mg/kg (1.54 and 2.36 μg/ml, respectively). However, it was still high for GW0742 0.3 mg/kg dose (1.45 μg/ml) (Table III). The highest concentration of GW0742 0.3 mg/kg was less than 0.1 μg/g tissue in the brain and liver. Thus, Kp value was less than 0.01. However, the concentration of the compound for 3 mg/kg and 30 mg/kg dose increased in the brain and liver with the highest concentrations 6 h after dosing (4.1 ± 0.98 and 6.98 ± 0.004, respectively, in the brain and 3.9 ± 0.78 and 6.75.± 0.74, respectively in the liver). To ensure that sufficient concentration of PPAR-β/δ agonist was attained to assess the neuroprotective properties of the compound in vivo, GW0742 0.3 mg/kg dose should be in further studies administered by intracerebrovascular infusion using Alzet miniosmotic pumps in subsequent in vivo studies. To confirm our results, we used to administrate intraperitoneally the 3 doses of GW0742 and determine the effect of GW0742 against cerebral ischemic injury.

Determination of GW0742 fractional occupancy. Free unbound GW0742 concentrations in brain were measured. Unbound concentrations were defined as the product of the total concentrations and unbound fraction. Notably, as reported in Table IV, chromatographic method was applied for Kd determination of GW0742 for 2 ppar subtypes (-β/δ and-γ). Brain protein-free drug concentrations by the Kd of GW0742 ratios were determined. GW0742 exhibited agonistic EC50 values of 2.4 ± 1.3 μM (PPAR-γ) and 0.002 ± 0.001 μM (PPAR-β/δ). In addition, the dissociation constants of PPAR-γ and PPAR-β/δ were 2.42 ± 0.37 and 0.80 ± 0.08, respectively. The fractional occupancy of PPAR-β/δ reached approximately 70% for the dose of 3 mg/g and more than 80% for the dose of 30 mg/kg. However, as regards to PPAR-γ, the fractional occupancy reached around 50% for the dose of 3 mg/kg and more than 60% for the dose of 30 mg/kg. Our results showed that in addition of PPAR-β/δ, the concentration of GW0742 in the brain reached

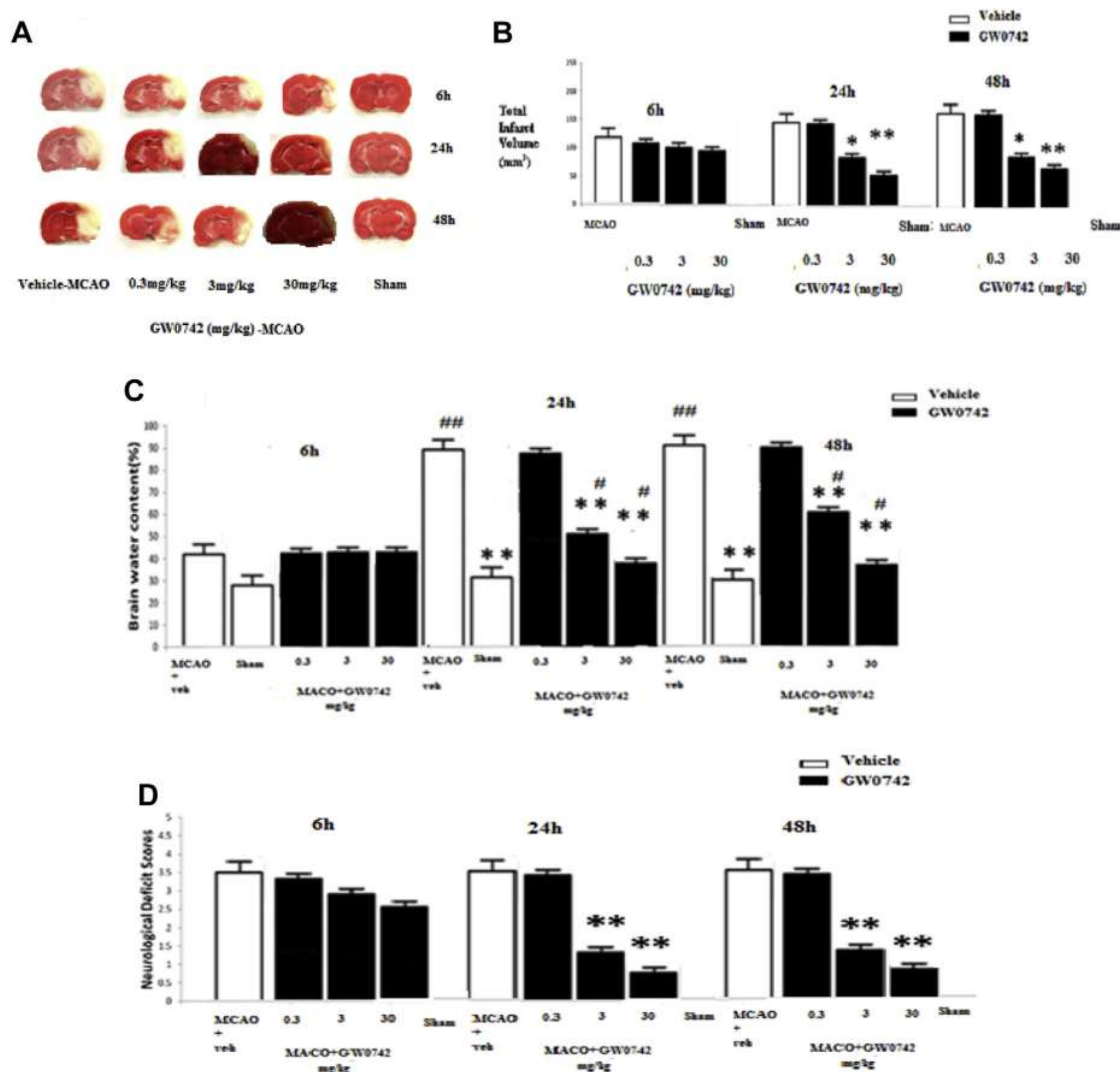

Fig 1. Effect of PPAR-β/δ agonist GW0742 treatment on infarction at 6, 24, and 48 hours after MCAO in mice. (**A**) Representative coronal brain sections (1-mm thick) through the territory of the MCA reveal infarcts in the vehicle-treated mice, GW0742-treated mice (0.3 mg/kg, 3 mg/kg, and 30 mg/kg), and absence of infarct in a sham surgery animal. Red colored regions in the TTC-stained sections indicate nonischemia, and pale-colored regions indicate ischemia portion of the brain. (**B**) The histogram shows the total infarct volume of mice for each group at different time-point. (**C**) The histogram shows the brain water content of mice. (**D**) The histogram shows the neurologic deficit scores of mice. Results are expressed as mean ± SEM. $^{*}P < 0.05$ vs MCAO Veh group; $^{**}P < 0.01$ vs MCAO Veh group; $^{\#}P < 0.05$ vs sham-operation group; and $^{\#\#}P < 0.01$ vs MCAO Veh group. MCAO, middle cerebral ischemia occlusion; SEM, standard error of the mean; TTC, 2,3,5- triphenyltetrazolium chloride. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

levels that triggered PPAR-γ responses. So, PPAR-γ might be involved in GW0742 neuroprotection action (Table IV).

PPAR-β/δ agonist GW0742 treatment improved the neurological deficits and decreased ischemic infarcted area and brain edema. To determine the neuroprotective effect of GW0742 against cerebral ischemic injury, we examined the infarct volume at 6, 24, and 48 hours after MCAO. Representative samples of TTC-stained brain sections are shown in Fig 1*A*, with corresponding infarction volumes and statistical data that are shown in Fig 1*B*. As shown in Fig 1*A*, there were no infract part in the sham-operated group. After 6 hours of MCAO, the vehicle-treated mice subjected

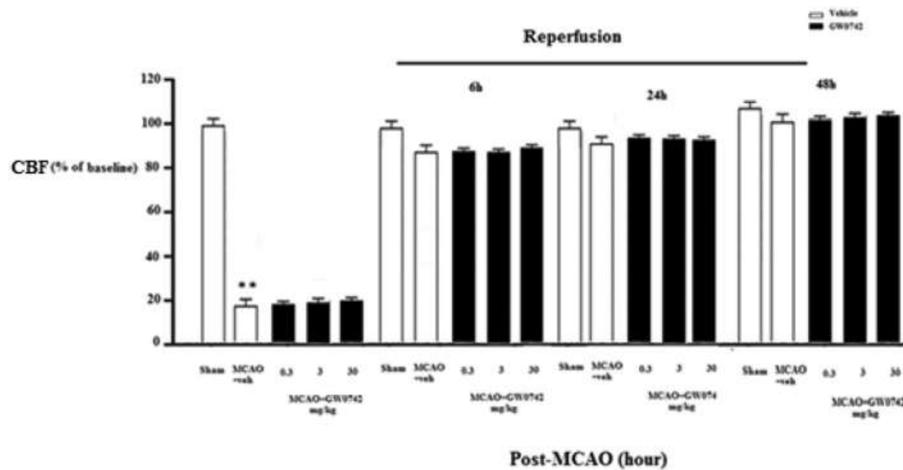

Fig 2. Effect of PPAR-β/δ agonist GW0742 on regional cerebral blood flow in mice with cerebral I/R. (A) Quantitative analysis of CBF in different groups. Data are expressed as mean ± SEM, n = 6. ***P* < 0.01 Sham vs MCAO Veh group. CBF, cerebral blood flow; I/R, ischemia–reperfusion; SEM, standard error of the mean; MCAO, middle cerebral artery occlusion.

to ischemia/reperfusion (I/R) injury exhibited a maximal infarct volume of 92.2 ± 4.17 mm^3. For the mice treated by different doses of GW0742, there was no significant difference in comparison with vehicle-treated mice. After 24 hours of MCAO, infarct volume was significantly increased in the vehicle-treated mice (152.2 ± 4.6 mm^3), whereas the 2 doses of GW0742 (3 and 30 mg/kg) reduced the infarct volumes induced by MCAO, which dropped to 85.2 ± 2.7 mm^3 and 49.1 ± 1.4 mm^3, respectively, with the highest inhibition level at a dose of 30 mg/kg vs vehicle group ($P < 0.01$). After 48 hours of MCAO, a significant reduction was also achieved by 2 doses of 3 and 30 mg/kg ($P < 0.05$ vs vehicle controls). Our results showed that GW0742 had a dose-dependent effect in affecting the infarct volume. When the dosage of GW0742 was 3 mg/kg or 30 mg/kg, the infarct volume was significantly reduced compared with vehicle starting at 24 hours after MCAO with the lowest volume for 30 mg/kg. The dose at 0.3 mg/kg did not reduce significantly the infarct volume compared with vehicle. In addition, at 24 and 48 hours post-I/R, brain water content was significantly increased in the MCAO vehicle treated group as compared with the sham-operation group ($P < 0.01$; Fig 1*C*). At 24 and 48 hours post-I/R, brain water content was significantly increased in the MCAO + GW0742 group as compared with the sham-operation group ($P < 0.05$). At 24 and 48 hours post-I/R, brain water content was significantly decreased in the MCAO + GW0742 group at a dose of 3 mg/kg and 30 mg/kg as compared with the MCAO + vehicle group ($P < 0.01$) with the lowest level for 30 mg/kg, whereas there was no significant difference at 6 hours ($P > 0.05$ Fig 1*C*). Neurological deficit was also evaluated. Consequently, obvious neurological deficit was observed in the MCAO group (Fig 1*D*), whereas the mice in the sham-operated group presented no neurologic deficit. Moreover, at 24 and 48 hours after reperfusion, the neurologic score of the group treated with 3 mg/kg or 30 mg/kg of GW0742 was significantly lower than those treated with vehicle ($P < 0.01$), indicating that GW0742 ameliorated the brain function starting 24 hours after cerebral ischemia.

Effect of MCAO and GW0742 on reperfusion CBF. To rule out the possibility that GW0742 might alter CBF, laser Doppler flowmetry was used to monitor cortical CBF in both vehicle- and GW0742-treated (0.3, 3, and 30 mg/kg) animals. A reduction of CBF by >80% of the baseline indicated a successful occlusion of the middle cerebral artery. Fig 2 showed changes in relative CBF during 1 hour of MCAO and 6, 24, and 48 hours of reperfusion in comparison with sham-operated group. However, no significative difference in CBF was found during or after MCA occlusion between animals (n = 6) that received the 3 doses of GW0742 or vehicle (Fig 2).

Effect of PPAR-β/δ agonist GW0742 on the hepatic protein level of inflammatory factors. To further investigate the potential concomitant reduction in the hepatic protein level of CXC chemokines, SAA-1, TNF-α, IL-1β, and IL-6 associated with PPAR-β/δ agonist GW0742 treatment, we measured these molecules in mouse samples at 6, 24, and 48 hours following MCAO. Compared with the vehicle sham group, the MCAO-vehicle model group exhibited a higher

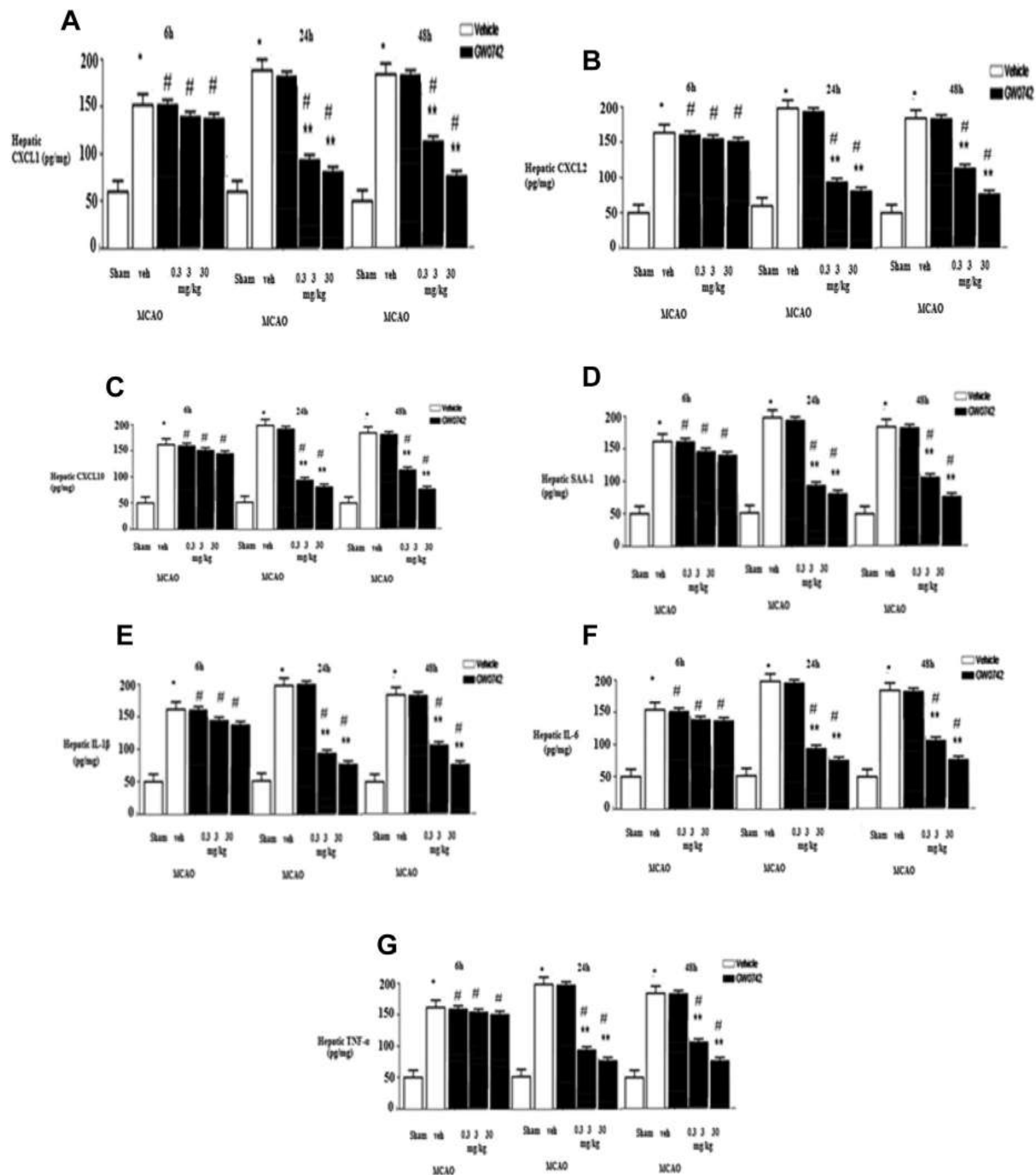

Fig 3. Effect of PPAR-β/δ agonist GW0742 treatment on the hepatic protein levels of inflammatory factors CXCL1 (**A**), CXCL2 (**B**), CXCL10 (**C**), SAA-1 (**D**), IL-1β (**E**), IL-6 (**F**), and TNF-α (**G**) in infarcted and sham-operated (Sham) mice at 6, 24, and 48 hours following MCAO (n = 6 in each treated group). Results are expressed as mean ± SEM. *$P < 0.01$, MCAO vs Sham group; **$P < 0.01$, MCAO-GW0742 vs MCAO Veh group; $^{\#}P < 0.01$, MCAO-GW0742 vs Sham Veh group. SAA1, serum amyloid A-1; IL-1β, interleukin-1β; IL-6, interleukin-6; TNF-α, tumor necrosis factor; SEM, standard error of the mean; MCAO, middle cerebral artery occlusion.

hepatic protein level of CXCL1, CXCL2, CXCL10, SAA-1, TNF-α, IL-1β, and IL-6 ($P < 0.01$). Brain ischemia/reperfusion significantly increased protein level of inflammatory factors at 6 hours with the peak time of expression at 24 hours. Whereas at 24 hours, GW0742 administration i.p. dose-dependently inhibited this increase. This inhibition persisted to 48 hours. A significant ($P < 0.01$) increase in CXC

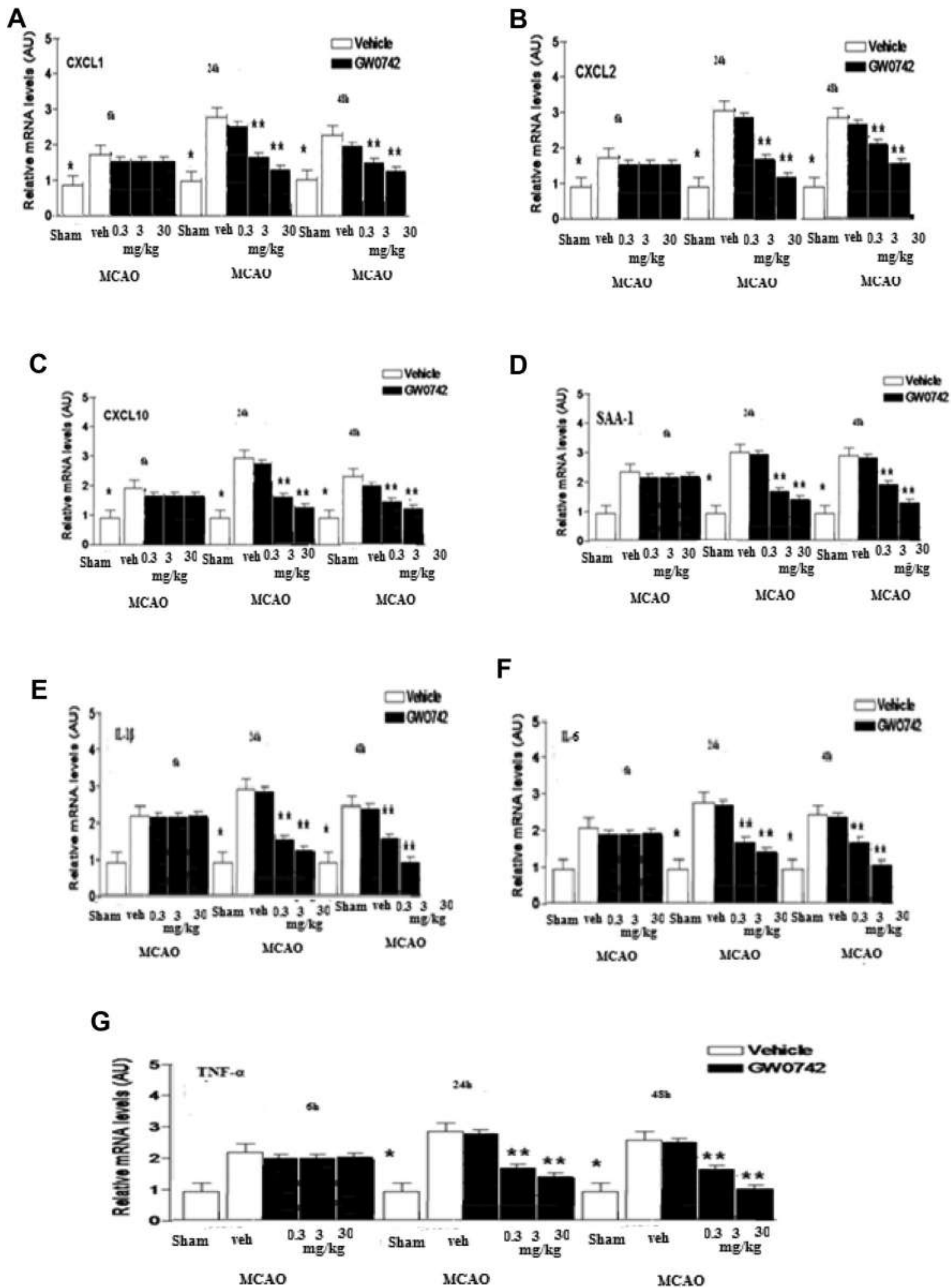

Fig 4. Liver mRNA expression of (**A**) CXCL1, (**B**) CXCL2, (**C**) CXCL10, (**D**) SAA-1, (**E**) IL-1β, (**F**) IL-6, and (**G**) TNF-α at 6, 24, and 48 hours following surgery in male C57 BL/6J wild-type mice treated with intraperitoneal (i.p.) doses of the PPAR-β/δ agonist GW0742 (0.3 mg/kg, 3 mg/kg, and 30 mg/kg) or vehicle (1.0% w/v. carboxymethylcellulose medium viscosity). GAPDH was used as an internal control. Results are expressed as mean ± SEM of 6 individual animals per group. Note that sham surgery alone induces the induction of all transcripts. *$P < 0.05$. Sham vs MCAO Veh group; **$P < 0.01$, MCAO-GW0742 vs MCAO Veh group. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; SAA1, serum amyloid A-1; IL-1β, interleukin-1β; IL-6, interleukin-6; TNF-α, tumor necrosis factor; SEM, standard error of the mean; MCAO, middle cerebral artery occlusion; mRNA, messenger RNA.

chemokines, SAA-1, TNF-α, IL-1β, and IL-6 protein levels was shown in cerebral ischemic mice, which was either treated with PPAR-β/δ agonist GW0742 (3 or 30 mg/kg) or a vehicle solution (1.0% w/v. carboxymethylcellulose medium viscosity) as compared with sham-operated mice. In addition, GW0742-treated mice with 3 or 30 mg/kg showed a significant reduction of CXC chemokines, SAA-1, TNF-α, IL-1β, and IL-6 protein levels as compared with MCAO-vehicle treated mice ($P < 0.01$) starting at 24 hours after reperfusion (Fig 3).

Effect of PPAR-β/δ agonist GW0742 on the hepatic mRNA expression levels of inflammatory factors following focal cerebral ischemia or sham surgery. After showing the GW0742 effect on hepatic protein level of inflammatory factors, we next analyzed whether the activation of PPAR-β/δ regulated CXCL1, CXCL2, CXCL10, SAA-1, TNF-α, IL-1β, and IL-6 hepatic gene expression at 6, 24, and 48 hours after reperfusion in MCAO–vehicle-treated mice, sham and MCAO-GW0742 treated group. Quantitative PCR revealed a significant upregulation in the hepatic expression levels of CXCL1, CXCL2, CXCL10, SAA-1, TNF-α, IL-1β, and IL-6 following the MCAO procedures compared to sham group at 6 hours after reperfusion with the peak time of expression at 24 hours. PPAR-β/δ agonist GW0742 treatment significantly reduced the expression of each of the acute phase reactants in the MCAO group in comparison with MCAO–vehicle-treated group starting at 24 hours after reperfusion with a dose of 3 mg/kg and 30 mg/kg ($P < 0.05$) (Fig 4). Importantly, GW0742 treatment acted broadly to suppress the expression of CXCL1, CXCL2, CXCL10, SAA-1, TNF-α, IL-1β, and IL-6.

PPAR-β/δ agonist GW0742 treatment reduced hepatic and brain neutrophil infiltration. Neutrophils infiltration in ischemic brain hemispheres was assessed at 6, 24, and 48 hours following MCAO. Brain ischemia was associated with a remarkable cerebral infiltration of neutrophils as compared with sham-operated group. In addition, PPAR-β/δ agonist GW0742 treatment (3 mg/kg or 30 mg/kg) reduced neutrophil cerebral infiltration within the ischemic brain hemispheres (Fig 5*A1–4*) as compared with vehicle-treated MCAO mice (Fig 5*A1*). Treatment with GW0742 significantly reduced the number of neutrophils in the brain parenchyma starting 24 hours following MCAO (Fig 5*A*). In the same animals, both sham and MCAO procedures increased the levels of neutrophils present in the liver after 24 hours (Fig 5*B1, B2*) and treatment with 3 mg/kg or 30 mg/kg dose of PPAR-β/δ agonist GW0742 had a significant impact on the number of neutrophils recruited to the liver after surgery (Fig 5*B4, B5*). The number of neutrophils present in the liver was shown to be a good surrogate for assessing the APR induction. PPAR-β/δ agonist GW0742-treated animals (30 mg/kg) contained fewer neutrophils compared to PPAR-β/δ agonist GW0742-treated animals with 3 mg/kg, whereas, a dose of 0.3 mg/kg has no significative effect (Fig 5*B*).

Effect of GW0742 on PPAR-β/δ mRNA and protein expression. Compared to sham experimental groups, PPAR-β/δ mRNA expression (Fig 6*A*) was significantly decreased in the vehicle-MCAO treated mice after 6, 24, and 48 hours of MCAO. Ischemia/reperfusion treatment with 3 mg/kg or 30 mg/kg dose of GW0742 significantly increased the expression of PPAR-β/δ mRNA in ischemic brain hemispheres after focal cerebral ischemia, with the peak time of expression at 24 hours for the dose of 30 mg/kg. The level of PPAR-β/δ mRNA expression in the 24 hour MCAO-GW0742 treated group was about 75% higher than that in the sham operation group (Fig 6*A*). Similar to the PPAR-β/δ mRNA expression, PPAR-β/δ protein expression in brain of the vehicle model group decreased significantly compared with that of the vehicle sham group ($P < 0.01$). The level of PPAR-β/δ protein expression in the 24 hour I/R-GW0742 treated group was about 65% higher than that in the sham operation group. Compared with that of the sham operation group, PPAR-β/δ protein expression in the I/R-treated group with 3 mg/kg or 30 mg/kg dose of GW0742 significantly increased, peaking at 24 hours after MCAO for the dose of 30 mg/kg. No significant difference was observed between the GW0742 0.3 mg/kg model group and the vehicle model group for both PPAR-β/δ mRNA and protein expression levels in each time-point ($P > 0.05$; Fig 6*B*).

PPAR-β/δ agonist GW0742 decreased BBB permeability. BBB leakage is the characteristic feature of cerebral ischemia/reperfusion injury, contributing to the formation of brain edema and hemorrhage. Based on the effect of the PPAR-β/δ agonist GW0742-induced reduction in brain edema in mice with cerebral ischemia/reperfusion injuries, the next experiment was performed to examine the effect of GW0742 on BBB integrity. Quantitative spectrometry detected extensive Evans Blue leakage in MCAO group compared with the sham group at 6, 24, and 48 hours after MCAO. As shown in Fig 7*A*, MCAO group showed a significant increase in BBB permeability, as compared with sham-operated group at 6, 24, and 48 hours. In addition, the results demonstrated that GW0742 (3 mg/kg or 30 mg/kg) significantly reduced the BBB permeability

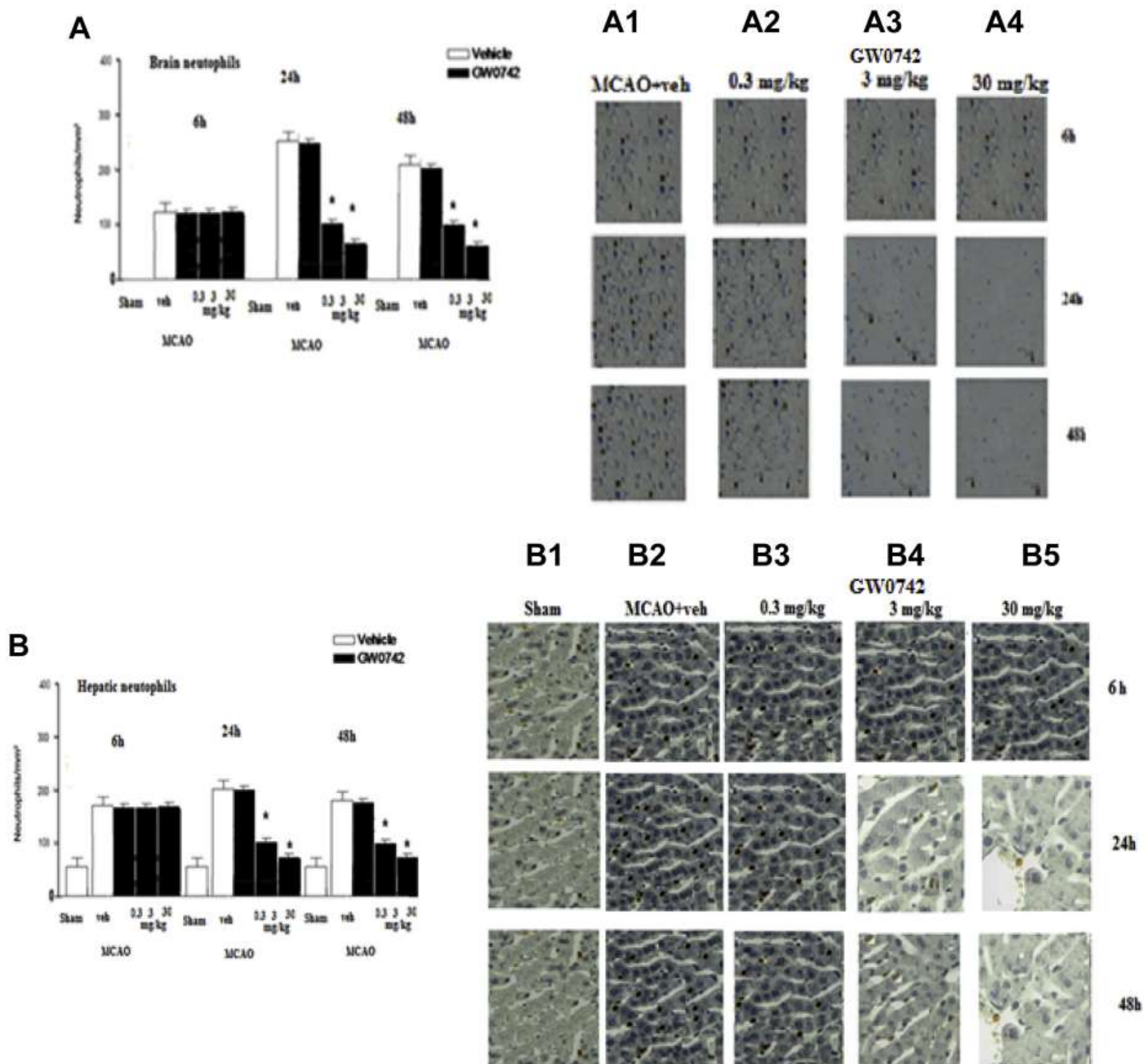

Fig 5. Representative images of cerebral and hepatic neutrophil infiltration after 6, 24, and 48 hours in sham-operated transient MCAO male C57 BL/6J wild-type mice treated with intraperitoneal (i.p.) doses of the PPAR-β/δ agonist GW0742 (0.3, 3, and 30 mg/kg) or vehicle (1.0% w/v. carboxymethylcellulose medium viscosity). (**A**) Left panel: quantification of infiltrated neutrophils per area in frozen sections of infarcted hemispheres at 6, 24, and 48 hours following MCAO (n = 6/group). Right panel: representative images of cerebral neutrophil infiltration at 6, 24, and 48 hours following MCAO in mice treated with (**A1**) vehicle or (**A2**) PPAR-β/δ agonist GW0742 (0.3 mg/kg), (**A3**) PPAR-β/δ agonist GW0742 (3 mg/kg), and (**A4**) PPAR-β/δ agonist GW0742 (30 mg/kg). The infarcted hemispheres are counterstained with 0.05% cresyl violet. (**B**) Left panel: quantification of infiltrated neutrophils in the liver of mice after 6, 24, and 48 hours (n = 6/group). Right panel: representative images of hepatic MBS1-stained neutrophils 6, 24, and 48 hours after sham surgery (**B1**) or transient MCAO (**B2, B3, B4,** and **B5**). The livers of animals treated with GW0742, (**B3, B4,** and **B5**), contained fewer neutrophils compared with vehicle-treated animals. The livers are counterstained with hematoxylin (blue). Treatment with PPAR-β/δ agonist GW0742 significantly reduced the number of neutrophils in the brain and the liver starting 24 hours following MCAO. Results are expressed as mean ± standard error of the mean. *$P < 0.05$. MCAO, middle cerebral artery occlusion. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

($P < 0.05$). Although, there was no significative difference between MCAO-vehicle treated mice and GW0742-treated mice with a dose of 0.3 mg/kg (Fig 7*A*).

PPAR-β/δ agonist GW0742 increased expression of zonula occludens-1 (ZO-1), Claudin-5, and occludin. To identify the relationship between TJ remodeling and BBB integrity, Western blotting was used to analyze

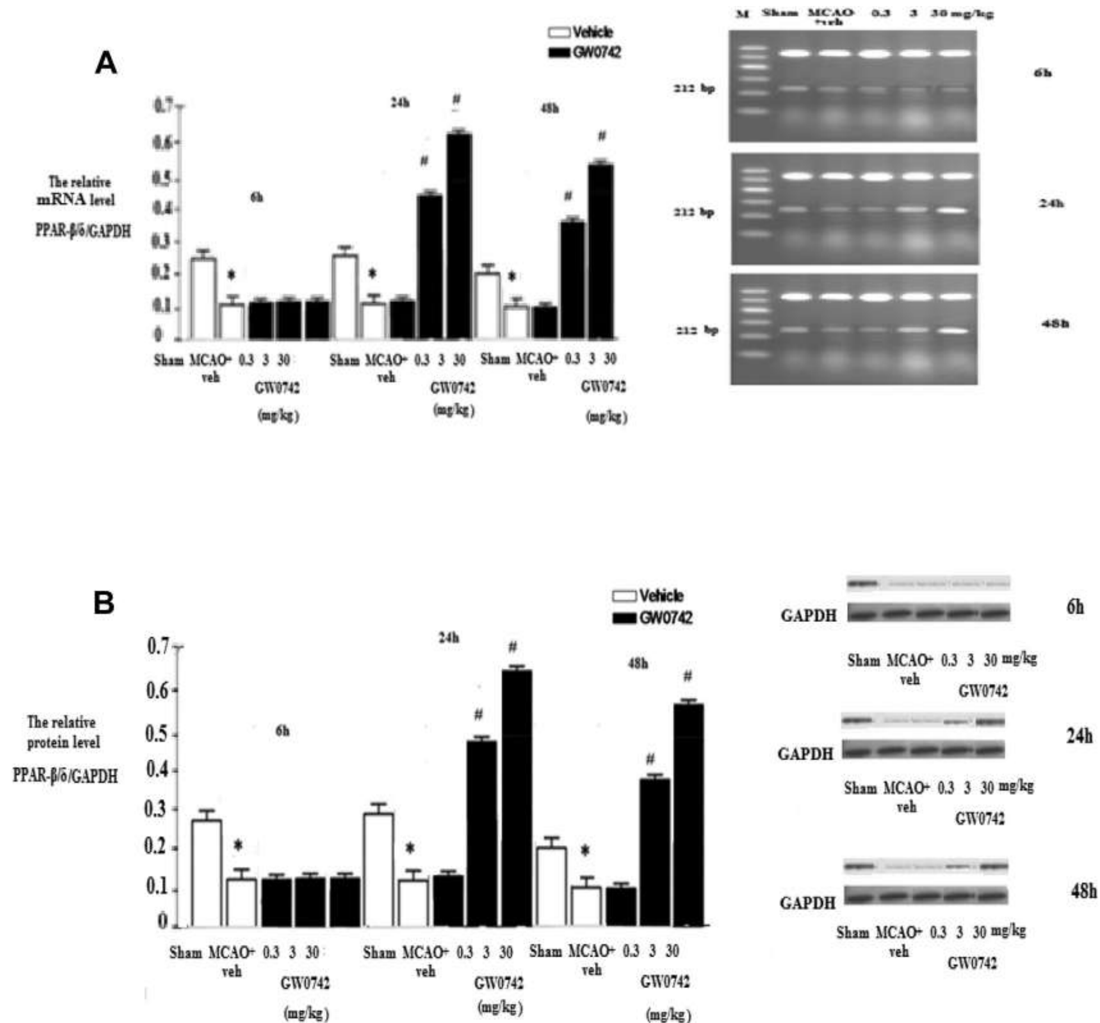

Fig 6. Effect of GW0742 on brain PPAR-β/δ mRNA and protein expression at 6, 24, and 48 hours following surgery in male C57 BL/6J wild-type mice treated with intraperitoneal (i.p.) doses of the PPAR-β/δ agonist GW0742 (0.3 mg/kg, 3 mg/kg, and 30 mg/kg) or vehicle (1.0% w/v. carboxymethylcellulose medium viscosity). (**A**) The relative mRNA level of PPAR-β/δ was normalized to GAPDH mRNA for each sample. (**B**) The protein level of PPAR-β/δ was calculated as ratios of the corresponding GAPDH protein level for each sample. Dates are expressed as mean ± SEM of 6 individuals per group. $^{*}P < 0.01$ MCAO-veh vs sham group; $^{\#}P < 0.01$, MCAO-GW0742 vs Sham Veh group. M, DNA marker; mRNA, messenger RNA; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MCAO, middle cerebral artery occlusion; mRNA, messenger RNA.

the expression of TJ proteins. Western blot analysis (Fig 7*B–D*) showed that the expression of ZO-1, Claudin-5, and occludin was decreased in the vehicle-treated MCAO mice at 6, 24, and 48 hours, whereas it increased significantly in response to GW0742 treatment (3 mg/kg or 30 mg/kg; $P < 0.01$) with the peak expression at 24 hours for 30 mg/kg. The impairment of tight junctions plays the key role in BBB opening, and it was observed that the expression of ZO-1, Claudin-5, and occludin, tight junction proteins, was significantly elevated in the ischemia/reperfusion-injured brains in the GW0742 treatment group with a dose of 30 mg/kg compared with that in the vehicle group. Although, there was no significative difference between MCAO-vehicle and GW0742 treated mice with a dose of 0.3 mg/kg. These data indicated that GW0742 could significantly reduce BBB permeability during cerebral ischemia/reperfusion injury starting 24 hours after MCAO.

Effect of GW0742 on MMP-9 mRNA and protein expression. At 6, 24, and 48 hours post-I/R, MMP-9 mRNA expression was increased in both the MCAO-vehicle treated mice and MCAO + GW0742 groups as compared with the sham-operation group ($P < 0.01$) with the highest expression achieved at

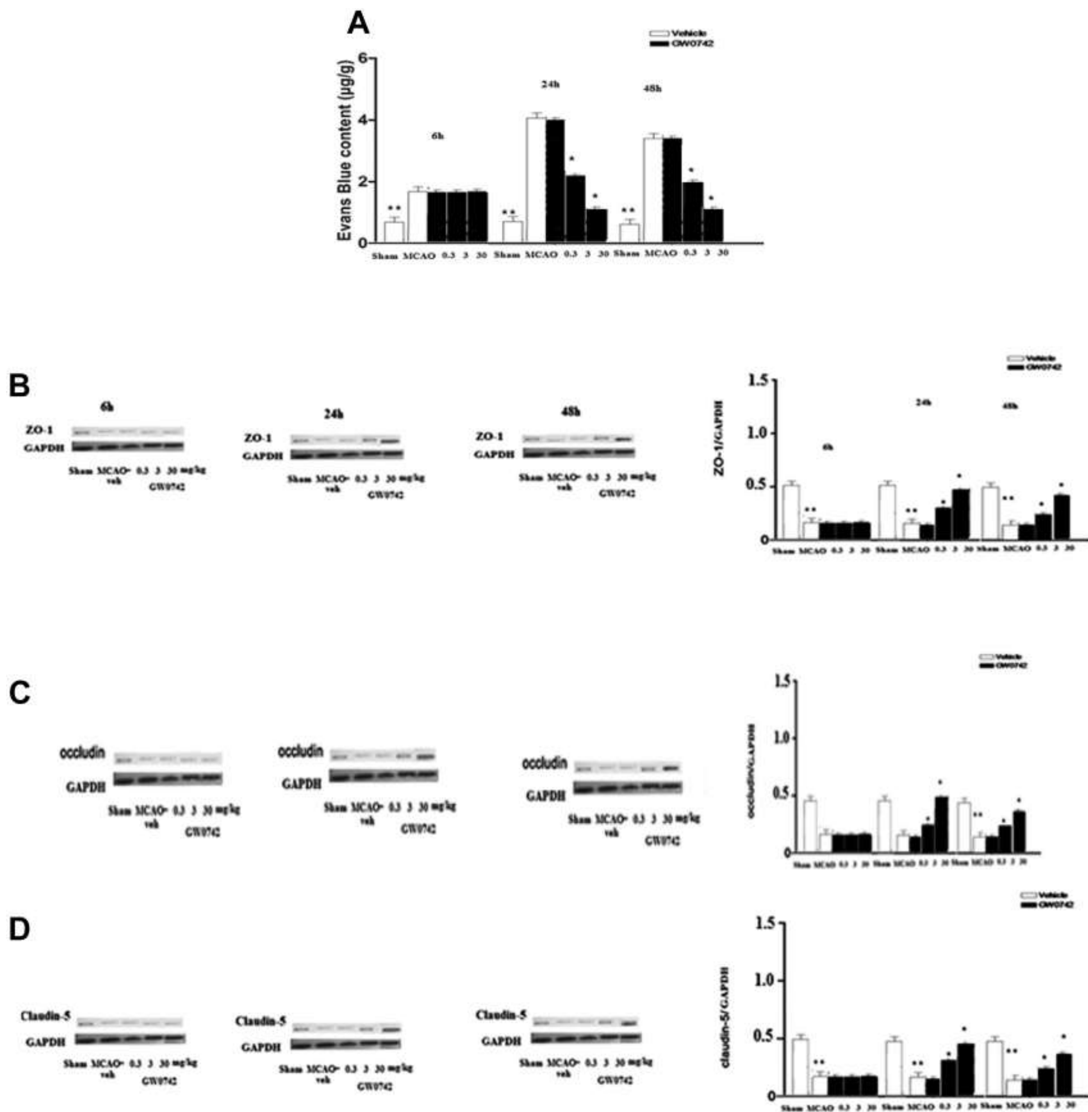

Fig 7. Effect of PPAR-β/δ agonist GW0742 on blood–brain barrier permeability and tight junction impairment in brains of sham-operated and MCAO male C57 BL/6J wild-type mice treated with intraperitoneal (i.p.) doses of the PPAR-β/δ agonist GW0742 (0.3 mg/kg, 3 mg/kg, and 30 mg/kg) or vehicle (1.0% w/v. carboxymethylcellulose medium viscosity). (**A**) Quantitative analysis of EB extravasation by spectrophotometry. Bar graph shows a quantification analysis of Evans blue contents in brain tissue (n = 6 per group). Data are expressed as mean ± SEM. *$P < 0.01$, vehicle vs treated MCAO group, **$P < 0.01$ vs the sham group. Representative result of ZO-1 (**B**), occludin (**C**), and Claudin-5 (**D**) expression at 6, 24, and 48 hours after MCAO. EB, Evans Blue; I/R, ischemia–reperfusion; SEM, standard error of the mean; MCAO, middle cerebral artery occlusion.

24 hours (Fig 8*A*). At 24 and 48 hours post-I/R, MMR-9 mRNA expression in the MCAO + GW0742 groups (3 mg/kg or 30 mg/kg) was lower than that of the MCAO group ($P < 0.01$), with the lowest expression for a dose of 30 mg/kg, whereas there was no significant difference at 6 hours ($P > 0.05$; Fig 8*A*). At 6, 24, and 48 hours, MMP-9 protein expression in the MCAO + GW0742 groups (3 mg/kg or 30 mg/kg) was significantly lower than that in the MCAO group ($P < 0.01$), whereas there was no significant difference at 6 hours ($P > 0.05$; Fig 8*B*). Notably, these MMP-9 protein expression findings match the MMP-9 mRNA expression findings stated in previous section. Increased MMP 9 levels were observed 6 hours after MCAO and continued up to 24 hours.

Effect of GW0742 on MMP-9 activity. Gelatin zymography analysis revealed a small amount of MMP-9 activity in the sham-operation group (Fig 9). In contrast, at 6, 24, and 48 hours post-I/R, MMP-9 enzyme activity was significantly increased in both

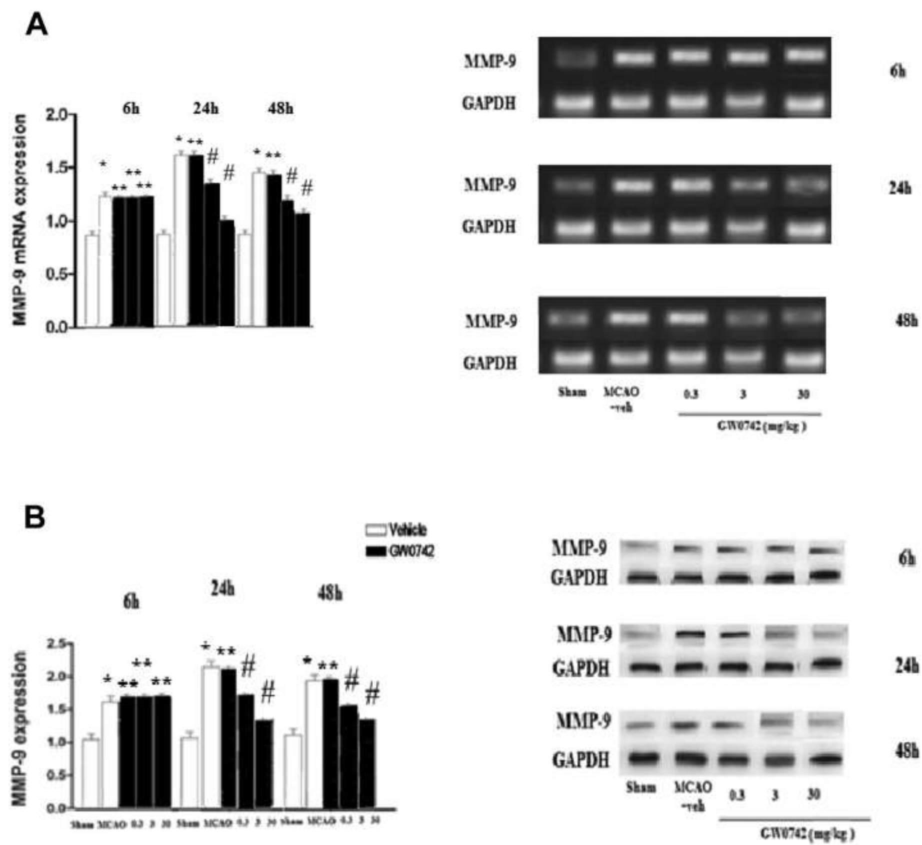

Fig 8. MMP-9 mRNA and protein expression levels in the ischemic zone of the mice brain. (**A**) MMP-9 mRNA expression level in the ischemic zone of the mice brain was determined by RT-PCR and densitometric analysis with GAPDH used as an internal control. (**B**) MMP-9 protein expression levels in the ischemic zone of the mice brain were determined by Western blotting and densitometric analysis with GAPDH used as an internal control. Values are expressed as means ± SEM (n = 6 per group); *$P < 0.01$ vs the sham group, **$P < 0.01$ vs the sham group; $^{\#}P < 0.01$ vs the MCAO group. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; SEM, standard error of the mean; MCAO, middle cerebral artery occlusion; mRNA, messenger RNA.

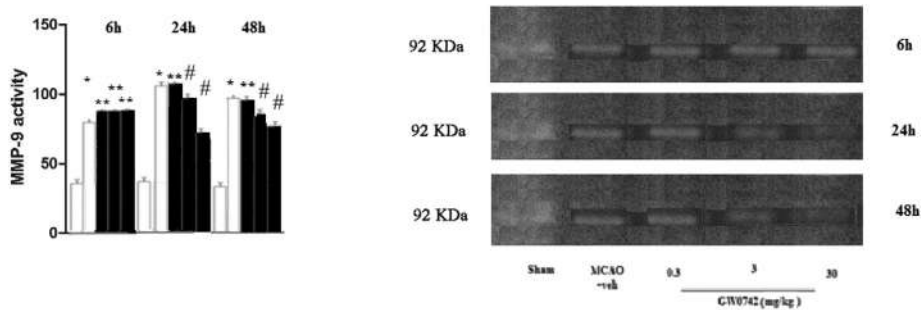

Fig 9. MMP-9 activity in the ischemic zone of the mice brain was determined by gelatin zymography and densitometric analysis. Values are expressed as means ± SEM (n = 6 per group); *$P < 0.01$ vs the sham group, **$P < 0.01$ vs the sham group, and $^{\#}P < 0.01$ vs the MCAO group. SEM, standard error of the mean; MCAO, middle cerebral artery occlusion.

the MCAO and MCAO + GW0742 groups as compared with the sham-operation group ($P < 0.01$) At 6, 24, and 48 hours post-I/R, MMP-9 activity was significantly lower in the MCAO + GW0742 (3 mg/kg or 30 mg/kg) groups as compared to the MCAO group ($P < 0.01$), whereas there was no significant difference at 6 hours ($P > 0.05$; Fig 9). These results suggested that GW0742 decreased the MMP activity.

Table V. Correlations between inflammatory markers and clinical outcomes

Clinical outcomes (rows) / Inflammatory variables (columns)		CXCL1 (pg/mg)	CXCL2 (pg/mg)	CXCL10 (pg/mg)	SAA-1 (pg/mg)	TNF-α (pg/mg)	IL-1β (pg/mg)	IL-6 (pg/mg)
Infarct volume (mm^3)	Correlation coefficient (P-value)	r = 0.141 (0.032)*	r = 0.171 (0.032)*	r = 0.141 (0.032)*	r = 0.251 (0.032)*	r = 0.321 (<0.01)**	r = 0.341 (<0.01)**	r = 0.301 (<0.01)**
		r = 0.231 (0.022)*	r = 0.131 (0.042)*	r = 0.231 (0.042)*	r = 0.239 (0.046)*	r = 0.313 (<0.01)**	r = 0.331 (<0.01)**	r = 0.321 (<0.01)**
		r = 0.223 (0.024)*	r = 0.223 (0.038)*	r = 0.223 (0.024)*	r = 0.233 (0.042)*	r = 0.323 (<0.01)**	r = 0.323 (<0.01)**	r = 0.317 (<0.01)**
BBB leakage (ug/g)	6 h post I/R	r = 0.181 (0.032)*	r = 0.151 (0.036)*	r = 0.141 (0.039)*	r = 0.141 (0.031)*	r = 0.241 (<0.01)**	r = 0.251 (<0.01)**	r = 0.241 (<0.01)**
	24 h	r = 0.191 (0.042)*	r = 0.141 (0.041)*	r = 0.151 (0.032)*	r = 0.191 (0.044)*	r = 0.231 (<0.01)**	r = 0.231 (<0.01)**	r = 0.234 (<0.01)**
	48 h	r = 0.223 (0.022)*	r = 0.136 (0.032)*	r = 0.133 (0.030)*	r = 0.143 (0.042)*	r = 0.223 (<0.01)**	r = 0.222 (<0.01)**	r = 0.253 (<0.01)**
MMP-9 expression		r = 0.161 (0.032)*	r = 0.191 (0.036)*	r = 0.161 (0.042)*	r = 0.141 (0.032)*	r = 0.279 (0.001)**	r = 0.341 (0.002)**	r = 0.248 (0.003)**
		r = 0.166 (0.046)*	r = 0.161 (0.043)*	r = 0.145 (0.044)*	r = 0.131 (0.041)*	r = 0.245 (0.004)**	r = 0.361 (0.001)**	r = 0.241 (0.002)**
		r = 0.256 (0.029)*	r = 0.165 (0.042)*	r = 0.163 (0.039)*	r = 0.223 (0.039)*	r = 0.233 (0.003)**	r = 0.323 (0.002)**	r = 0.223 (0.001)**
TJ proteins expression		r = −0.191 (0.032)*	r = −0.241 (0.032)*	r = −0.241 (0.042)*	r = −0.251 (0.042)*	r = −0.351 (0.002)**	r = −0.441 (0.002)**	r = −0.341 (0.001)**
		r = −0.231 (0.048)*	r = −0.231 (0.042)*	r = −0.231 (0.032)*	r = −0.221 (0.045)*	r = −0.331 (0.003)**	r = −0.431 (0.001)**	r = −0.311 (0.002)**
		r = −0.323 (0.042)*	r = −0.223 (0.022)*	r = −0.253 (0.022)*	r = −0.226 (0.042)*	r = −0.323 (0.002)**	r = −0.423 (0.002)**	r = −0.303 (0.002)**
Hepatic neutrophil infiltration		r = 0.141 (0.022)*	r = 0.151 (0.039)*	r = 0.161 (0.032)*	r = 0.191 (0.029)*	r = 0.256 (0.003)**	r = 0.291 (0.032)**	r = 0.311 (0.002)**
		r = 0.131 (0.034)*	r = 0.191 (0.042)*	r = 0.139 (0.041)*	r = 0.176 (0.041)*	r = 0.261 (0.002)**	r = 0.251 (0.042)**	r = 0.351 (0.001)**
		r = 0.223 (0.032)*	r = 0.233 (0.032)*	r = 0.225 (0.042)*	r = 0.219 (0.032)*	r = 0.292 (0.002)**	r = 0.203 (0.022)**	r = 0.323 (0.002)**
Brain neutrophil infiltration		r = 0.151 (0.032)*	r = 0.163 (0.032)*	r = 0.191 (0.022)*	r = 0.141 (0.032)*	r = 0.341 (<0.01)**	r = 0.441 (<0.01)**	r = 0.336 (0.002)**
		r = 0.169 (0.042*)	r = 0.151 (0.042)*	r = 0.161 (0.042)*	r = 0.131 (0.041)*	r = 0.331 (<0.01)**	r = 0.431 (<0.01)**	r = 0.391 (0.001)**
		r = 0.224 (0.037)*	r = 0.224 (0.022)*	r = 0.213 (0.032)*	r = 0.223 (0.022)*	r = 0.323 (<0.01)**	r = 0.423 (<0.01)**	r = 0.323 (0.003)**

Abbreviations: BBB, Blood–brain barrier; *IL-1β*, interleukin-1β; *IL-6*, interleukin-6; *TJ*, tight junction; *TNF*, tumor necrosis factor alpha.
*$P < 0.05$, **$P < 0.01$.

Correlation. As shown in Table IV, the correlations between inflammatory mediators, such as cytokines and acute-phase reactants and clinical outcomes were calculated at 6, 24, and 48 hours post I/R. Pearson's correlation coefficients were significant for all the inflammatory variables. Indeed, the results showed a significant negative correlation between the chemokines and cytokines and TJ proteins expression ($P < 0.05$, $P < 0.01$, respectively). However, there were significant positive correlations between all inflammatory markers and the other clinical outcomes such as infarct volume, BBB permeability, MMP-9 expression, and hepatic and brain neutrophil infiltration (Table V).

DISCUSSION

The acute response to brain injury was manifested by increased synthesis of hepatic CXC chemokines which increased neutrophil levels in the blood, liver, and brain.[5] Cerebral ischemia is commonly accompanied by inflammation, and reperfusion after brain ischemia increases the inflammatory reactions, which can worsen neuronal injury.[3] Specifically, a substantial and persistent production of proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6 could significantly increase the risk and extent of brain injury.[35] Experiments in vivo showed a neuroprotective effect of GW0742 when animals were treated before or after the induction of focal or global cerebral ischemia. However, it is not known if GW0742 is protective during reperfusion. In the present study, we investigated if acute GW0742 treatment 10 minutes before post-ischemic reperfusion would improve infarction and edema formation during cerebral ischemia. Thus, our study was directed to clarify the therapeutic time window of PPAR-β/δ agonist GW0742 in stroke animal model by treating with different doses and to evaluate, for the first time, the effect of the reduction of hepatic APR following brain ischemia. The data presented in this report demonstrated that GW0742 administered 10 minutes before post-ischemic reperfusion, reduced infarct volume up to 65% independently of any alterations in CBF and other physiological parameters. Although GW0742 is a PPAR-β/δ agonist, it has not been investigated previously whether GW0742 could indeed activate the PPAR-β/δ receptors in the brain. Furthermore, it is unknown whether the neuroprotective effects of GW0742 in the MCAO model are mediated via the activation of PPAR-β/δ. It has been reported that GW0742 displayed 300- to 1000-fold selectivity for PPAR-β/δ vs other PPARs in cell culture and animal models.[36] However, whether it can regulate PPAR-β/δ following brain ischemia is still unknown. In our study, we found that after GW0742 treatment, PPAR-β/δ mRNA and protein level were upregulated. However, we could not confirm that this effect indicated the activation of PPAR-β/δ. Further investigations should be performed to examine the specific DNA-binding activity of PPAR-β/δ and determine the PPAR-β/δ transcriptional activity in nuclear proteins extracted from brain tissues treated with GW0742 as described.[37] Many other groups have investigated GW0742 with respect to different nuclear receptor binding using a variety of assays. These included PPAR-α, PPAR-γ, and PPAR-β/δ using cell-based transactivation assays. Indeed, GW0742 was able to activate transcription mediated by only PPAR-α, PPAR-γ, and PPAR-β/δ among the nuclear receptors tested.[38] In our study, GW0742 exhibited agonistic EC50 values of 2.4 $\pm$ 1.3 μM (PPAR-γ) and 0.002 $\pm$ 0.001 μM (PPAR-β/δ). Moreover, the fractional occupancy of PPAR-β/δ reached more than 70%. We also found that it reached around 50% and more than 60% when the free unbound concentration was below the kd of the PPAR-γ receptor. Based to our data, the concentration of GW0742 in the brain reached levels that triggered PPAR-β/δ. Considering these results, it appeared that in addition to PPAR-β/δ, GW0742 also binded to PPAR-γ, and activation of this isoform of PPARs could afford neuroprotection from ischemic injury by anti-inflammatory mechanism. Thus, in the present study, we could not exclude the possibility that PPAR-γ activation was involved in the actions of GW0742. It is also interesting to note that although GW0742 seemed to have a clear dose response on PPAR-β/δ mRNA/protein in most of the biochemical assays, the 30 mg/Kg and 3 mg/Kg had little or no difference suggesting that levels of PPAR-β/δ regulation was not solely responsible for the biochemical changes. Future studies are needed to determine the exact signaling pathway central for the action of GW0742 in ischemic injury of the brain. It was known that activation of PPAR-β/δ or-γ resulted in its binding to specific PPAR response elements in the promoter regions of various specific target genes, which in turn leaded to either activation or suppression of target genes.[39] Significative positive correlations between inflammatory proteins and worse clinical outcomes were established. The protective effect of GW0742 against ischemic brain injury was likely attributable, at least in part, to its anti-inflammatory properties. In the present study, we demonstrated that GW0742 markedly reduced several proinflammatory cytokines levels that were thought to contribute to ischemic brain injury. We also showed that GW0742 treatment has therapeutic effect on acute expression of inflammatory genes in the liver in response to inflammation in the brain. Indeed, administration of

PPAR-β/δ agonist GW0742 significantly reduced hepatic CXC chemokines. To our knowledge, this is the first study to demonstrate that markers of the APR were inhibited by the administration of GW0742. After having confirmed that CXC chemokines and SAA-1 were markedly upregulated in the liver in response to ischemic brain injury, we showed that these changes were associated with increased levels of hepatic and brain neutrophils. Treatment with GW0742 reduced brain and hepatic neutrophil recruitment following ischemic brain injury. The precise mechanism underlying the latter effect was unclear, but might occur via the PPAR-mediated repression of NF-κB. Numerous experimental studies proved that activation of NF-κB was implicated in stroke. NF-κB plays a central role in the regulation of many genes responsible for the generation of mediators or proteins in inflammation by binding to specific elements in their promoter region. These included the genes for TNF-α, IL-1β, and IL-6.[40] Taken together, the results suggested that the anti-inflammatory actions of GW0742 might play a key role in its protective effect against ischemic/reperfusion injury. Our findings were consistent with other studies in which GW0742 could exert its anti-inflammatory effect. Abundant evidence has confirmed that the disruption of BBB and the consequent brain edema were major contributors to the pathogenesis of ischemic stroke. Therefore, approaches focusing on the subsequent damage of BBB dysfunction should be considered. Mechanistically, we revealed that GW0742 treatment reduced the disruption of the tight junction via increasing ZO-1, occludin, and claudin-5 protein expression after 24 hours post-I/R, reduced the expression and activity of MMP-9 after 24 hours post-I/R, and attenuated the increase in BBB permeability after 24 hours post-I/R. Notably, the timing of the increases in TJs protein expression observed with GW0742 treatment (after 24 hours post-I/R) coincided with the timing of the reductions in the expression and activity of MMP-9 (after 24 hours post-I/R) and the improvement in BBB permeability (after 24 h post-I/R), suggesting that positive effects of GW0742 on BBB permeability post-I/R injury worked through MMP-9-mediated upregulation of ZO-1, occludin, and claudin-5 expression. The ability of GW0742 to provide direct neuroprotection had yielded some contradictory results and might vary depending on treatment times and exposure concentrations. There are some limitations in our data. Our dose response indicated that choice of GW0742 dose was critical in achieving protection in stroke models. First, because we found that GW0742 was protective at doses of 3 mg/kg and 30 mg/kg and that the protective actions of GW0742 were lost when the dose was decreased below that. Second, there was no clear and proportional relationship between the doses (3 and 30 mg/Kg) and the tissue levels, and it was hard to calculate whether there was real significant difference in brain levels at each time point between the doses. The choice of dose tested in a clinical trial will likely be crucial to the success of translating the promising protective effects of GW0742 in animals into humans. However, it should be noted that further studies are needed to confirm our findings for 2 reasons; in one side, because our data lacked clinical relevance regarding the therapeutic time windows and patients that are comply with such model are not to be found. On the other side, our data showed that effective timing of drug administration depended not only on the time elapsed since artery occlusion but also the time relative to reperfusion. In clinical practice, reperfusion could occur spontaneously; however, time of reperfusion was increasingly controlled by the use of thrombolytics and mechanical devices. To date, thrombolysis is the only FDA approved treatment for ischemic stroke. The practice is to administer thrombolytics and then determine if patients are candidates for neuroprotective trials. Our findings indicated that this approach might not be optimal, especially for therapies targeting events during reperfusion.

CONCLUSIONS

The present study demonstrated an associated increase in hepatic and central neutrophil recruitment following ischemic brain injury and that treatment with GW0742 reduced levels of hepatic CXCL1, CXCL2, CXCL10, and SAA-1 and proinflammatory cytokines such as TNF-α, IL-1β and IL-6, which subsequently reduced cerebral infarction. These results suggested the neuroprotective effect of GW0742 in MCAO model of mice. In addition, here, we showed for the first time that GW0742 treatment decreased BBB permeability in mice with cerebral I/R injury. Future studies are needed to determine the exact signaling pathway central for the action of GW0742 in brain ischemic injury.

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