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ORIGINAL RESEARCH

Microglia-Derived Adiposomes are Potential Targets for the Treatment of Ischemic Stroke

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Abstract

It is known that cerebral ischemia can cause brain inflammation and adiposome can serve as a depot of inflammatory mediators. In the study, the pro-inflammatory and pro-death role of adiposome in ischemic microglia and ischemic brain was newly investigated. The contribution of PPARγ to adiposome formation was also evaluated for the first time in ischemic microglia. Focal cerebral ischemia/reperfusion (I/R) animal model and the in vitro glucose-oxygen-serum deprivation (GOSD) cell model were both applied in the study. GOSD- or I/R-induced adiposome formation, inflammatory activity, cell death of microglia, and brain infarction were, respectively, determined, in the absence or presence of NS-398 (adiposome inhibitor) or GW9662 (PPARγ antagonist). GOSD-increased adiposome formation played a critical role in stimulating the inflammatory activity (production of TNF-α and IL-1β) and cell death of microglia. Similar results were also found in ischemic brain tissues. GOSD-induced PPARγ partially contributed to the increase of adiposomes and adiposome-mediated inflammatory responses of microglia. Blockade of adiposome formation with NS-398 or GW9662 significantly reduced not only the inflammatory activity and death rate of GOSD-treated microglia but also the brain infarct volume and motor function deficit of ischemic rats. The pathological role of microglia-derived adiposome in cerebral ischemia has been confirmed and attributed to its pro-inflammatory and/or pro-death effect upon ischemic brain cells and tissues. Adiposome and its upstream regulator PPARγ were therefore as potential targets for the treatment of ischemic stroke. Therapeutic values of NS-398 and GW9662 have been suggested.

Keywords Adiposome · Microglia · Inflammation · PPARγ · Cerebral ischemia

Introduction

Cerebral ischemia-induced stroke is the most prevailing cerebrovascular disease in the world, the third leading cause of death and the most frequent cause of permanent disability worldwide (Donnan et al. 2008). Inflammation plays an important role in the pathogenesis of ischemic stroke and other forms of brain injury (Jin et al. 2010). Microglia are multifunctional in brain. They may serve as scavengers or release growth factors to protect brain from ischemic insult (Lee et al. 2004; Lu et al. 2005). On the other hand, microglia may also participate in the pathogenesis of stroke or many other neurodegenerative diseases by releasing pro-inflammatory mediators, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6, nitric oxide (NO), and reactive oxygen species (ROS) (Jin et al. 2010). Inflammatory potential of microglia and inflammatory mediators released from these cells therefore become the major targets for the intervention of ischemic stroke. We have recently

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discovered that adiposome number was increased in microglia in response to the in vitro ischemia (glucose-oxygen serum deprivation or GOSD). The pathophysiological meaning and the molecular mechanism(s) responsible for this increase were still unclear.

Adiposome (or lipid droplets) is a lipid-rich cytoplasmic organelle composing of a neutral lipid core surrounded by monolayer membrane derived from endoplasmic reticulum (ER) or other membrane sources (Hu et al. 2017). Adiposome-associated proteins including, perilipin-1 (PLIN1), adipose differentiation-related protein (ADRP), and mannose-6-phosphate receptor binding protein 1 (MPRBP1 or PLIN3), all integrate into the phospholipid hemimembrane to form adiposome (Khatchadourian et al. 2012). In adipocytes, the expression level of ADRP is positively correlated with the amount of adiposomes and triacylglycerols (Xu et al. 2005). Adiposomes play a critical role in the regulation of lipid metabolism and energy maintenance because they are the major organelles for storage and turnover of neutral lipids (triglycerides/TGs and esterified fatty acids), particularly in adipocytes (Muller et al. 2010). They are also found in other cell types and have various functions including, cell signaling, activation of leukocytes, membrane trafficking, cancer development, histone storage, and as reservoirs of inflammatory mediators (Pope et al. 2016). Although adiposomes have been reported to involve in many inflammatory diseases (Kuroda and Sakaue 2017), their role(s) in cerebral ischemia was rarely reported before.

Peroxisome proliferator-activator receptor γ (PPARγ) belongs to the nuclear receptor family and is known to play a critical role in the regulation of adipogenesis, lipid metabolism, cell growth, apoptosis, and inflammation (Hong and Tontonoz 2008). Recently, PPARγ-regulated adipogenesis, lipid metabolism, and lipid storage have been linked to adiposome formation (Sauvant et al. 2011). It is believed that obesity-increased free fatty acids may act as PPARγ ligands (Ducharme and Bickel 2008) to regulate the expression of adipogenesis genes and formation of adiposome in adipocytes to down-regulate obesity. PPARγ has been reported to trigger the adiposome formation in colorectal cancer cells and oleic acid-treated NMuli liver cell (Assumpcao et al. 2017; Fan et al. 2009). We had previously demonstrated that PPARγ can be increased in neurons by GOSD to protect neurons from GOSD-caused cell injury (Wang et al. 2009). Could ischemic stress act through PPARγ to promote the adiposome formation and then inflammatory activity of microglia was worthy to know. In the study, the roles of microglia-derived adiposomes in the regulation of inflammation and death of microglia or even neurons were examined under the in vitro (GOSD) and in vivo ischemic condition, using a chemical blocking strategy with NS-398 (adiposome inhibitor). The link between PPARγ and adiposome formation was further delineated in GOSD microglia. Therapeutic values of NS-398 and GW9662 (PPARγ inhibitor) in the control of cerebral ischemia were also evaluated in ischemic rats.

Materials and Methods

Animals

Eight-week-old male Sprague Dawley (S.D.) rats (250–330 g) were purchased from Biolasco (Taipei, Taiwan) and kept in a ventilated room (22 ± 2 °C) under a 12/12-h light–dark cycle control and with free access to food and water. The study was approved by the Institutional Animal Care and Use Committees of National Chung Hsing University. All animals were treated in a humane way, following the NIH guideline, "Guide for the Humane Care and Use of Laboratory Animals."

Primary Microglia and Neurons

Microglia were prepared from the brains of postnatal day 1 (P1) newborns and neurons from the brains of day 18 embryos (E18) of S.D. rats, based on our previous methods (Lin et al. 2016). Purity of these enriched brain cells was about 95%, confirmed by pre-staining cells with anti-CD11b (GeneTex) for microglia and anti-MAP-2 (Cell Signaling) sera for neurons, respectively.

Glucose-Oxygen-Serum Deprivation (GOSD)

The GOSD model used in the study was based on our previous methods (Wang et al. 2009). Briefly, brain cells were grown in glucose-serum free DMEM (D5030, Sigma) and deprived of oxygen. This is an in vitro ischemia model to mimic the environment under the cerebral ischemia in vivo.

Drug or Microglia-Derived Conditioned Medium (MCM) Treatment

To know the role of adiposome in the regulation of inflammatory activity and cell death of GOSD microglia and the potential of PPARγ in stimulating adiposome formation, microglia were pre-treated with NS-398 (adiposome and COX-2 inhibitor; 1, 10, or 100 μM; Millipore), SC-58125 (COX-2 inhibitor; 0.1, 1, or 10 μM; Sigma), GW9662 (PPARγ inhibitor; 1 or 10 μM; Sigma), or Rosiglitazone (ROSI; PPARγ agonist; 1 or 10 μM; Sigma) for 2 h, and then followed by GOSD treatment for another 2 h. In the end, cells were harvested and ready for the tests as indicated. In another set of experiments, microglia-derived conditioned medium (MCM) collected from the GOSD (2 h)-treated microglia, with or without the pre-treatment with NS-398 (1,

10, or 100 μM; 2 h), was each co-cultured with neurons for 6 h under the GOSD condition. Neuronal viability of each group was then determined in the end. Drug doses applied were based on previous reports, indicating that NS-398 (at 0.1–10 μM) and SC-58125 (at 0.1–10 μM) could block the adiposome formation and COX-2 synthesis, respectively (Bozza et al. 2002), and GW9662 (at 10-40μM) and ROSI (at 10–30 μM) can inhibit and stimulate the activity and protein expression of PPARγ, respectively (Hung 2014).

Trypan Blue Dye Exclusion Assay

Cell viability was determined by the trypan blue dye exclusion assay as previously described (Lee et al. 2004). Briefly, the treated cells were stained with 0.4% trypan blue solution for 4 min. Number of the viable transparent cells was counted under light microscope.

Oil Red O Staining

Oil Red O staining was used to determine the adiposome formation in both GOSD-treated microglia and brain tissues (slices) from ischemic rats according to our previous report (Hung 2014). Briefly, the treated cells (in 3 wells per group; each experiment was repeated at least 3 times) or brain slices (2 slices per rat and 3 rats per group) were fixed by 4% paraformaldehyde for 10–20 min, followed by Oil Red O (14.7 μM; dissolved in 60% of isopropanol; Sigma) staining for 30–60 min. Cells were then washed by 60% of isopropanol and counterstained by 25% hematoxylin for 30 s to locate the nucleus. Percent of adiposome (in red)-positive cell per filed was counted and averaged from 30 randomly selected microscopic fields per group under the light microscope (Carl Zeiss/LD32). The magnification is 400X.

Western Blot Analysis

The protein expression levels of ADRP, TNF-α, IL-1β, COX-2, and PPARγ were determined in the treated microglia, using the Western blot analysis as previous described (Lee et al. 2004). The dilution factor for anti-ADRP (Santa Cruz) was 1:3000, anti-TNF-α (Abcam), anti-IL-1β (Abcam) and anti-COX2 (Abcam) 1:2500, anti-PPARγ (Santa Cruz) 1:2000, anti-β-actin (Sigma) 1:10,000, HRP-conjugated anti-rabbit IgG (Jackson ImmunoResearch) 1:60,000, and HRP-conjugated anti-mouse IgG (Jackson ImmunoResearch) 1:10,000. Actin was used as a loading control. The enhanced chemiluminescence (ELC) substrate kit (Perkin Elmer) was used to view proteins on the blot membrane. The protein expression levels were semi-quantified (densitometric quantitation) using the photoshop CS3 and image J system. Number under each protein band represents fold of the expression ratio (target protein/loading control protein) of each group over the expression ratio of normal control group (as 1). Each experiment was repeated at least 3 times.

DCF Assay for ROS Release

The release of ROS from the treated microglia was determined by the 2,7-dichlorofluorescin (DCF) assay as previously described (Lin et al. 2016). Each number in the figures represents the fluorescence unit per group averaged from triplicates of each group to reflect the amount of ROS being released.

ELISA for the Measurement of TNF-α and IL-1β

The amounts of TNF-α and IL-1β released from the treated microglia were determined by using specific ELISA kits (USCN) and based on the protocol provided by the company. Briefly, microglia in 12-well plate were treated respectively in each group (3 wells per group; 5×10^4 cells/well) and the supernatants (contain TNF-α and IL-1β) collected from each group (3 wells) were distributed equally to 3×4 wells (200 μl/well) in 96-well plate. Optical density (O.D.) of each well was determined by using a microplate reader (Perkin Elmer/VICTOR2 V) at 450 nm. The content change of TNF-α or IL-1β in each group was determined based on the averaged O.D. from 12 wells per group.

Focal Cerebral Ischemia and Intracisternal Drug Injection

To know the impacts of ischemia/reperfusion upon brain infarct volume, formation of microglia-associated adiposomes and adiposome-associated expression of TNF-α and IL-1β in ischemic brain, a transit cerebral ischemia animal model was used according to our previous methods (Lin et al. 2016). Briefly, one week before the experiments were started, eight-week-old male S.D. rats were weighed and randomly separated into various groups including, Sham, I/R0h, I/R6h, I/R12h, and I/R24h group. Sample size of each group is 6 or 3 animals per group depending on the readout system being used. In general, animal size for TTC staining is 6 (Fig. 5a) or 3 (Fig. 6) and for Oil Red O and IHC staining is 3 per group. Animals in the I/R groups were subjected to the bilateral common carotid artery occlusion (CCAO) plus right middle cerebral artery occlusion (MCAO) for 90 min and followed by reperfusion for 0 h (I/R0h group), 6 h (I/R6h group), 12 h (I/R12h group), or 24 h (I/R24h group) as indicated. Sham group received the same surgery except the blood vessels were not ligated. The ischemic surgery was performed under the anesthesia, using chloral hydrate (350–400 mg/kg/i.p.) plus ketorolac (pain killer; 1 mg/kg/i.m.). During the anesthesia period, excessive mucus accumulated in animal's throat was checked and removed

(if any) by cotton swab to avoid respiratory problem and heat lamp was used to keep body temperature at 37 °C. At the end of each reperfusion time, animals were sacrificed via the CO_2 asphyxiation. Brain tissues were then collected and subjected to TTC (2, 3, 5-triphenyltetrazolium chloride), Oil Red O or IHC staining.

To evaluate the drug effect of NS-398 and GW9662 upon brain infarct volume and motor function deficit of ischemic rats, S.D. rats were randomly separated into 3 groups, I/R + Vehicle, I/R + NS-398, and I/R + GW9662 group (3 animals each), 1 week prior to the ischemic surgery. All the animals received 90 min of bilateral CCAO plus unilateral MCAO and followed by reperfusion for 24 h. Dimethyl sulfoxide (DMSO; 0.1%/rat), NS-398 (0.3 mg/kg), and GW9662 (0.1 mg/kg) were individually injected into the cisterna magna of ischemic brain (15 µl/rat), 10 min prior to reperfusion began. DMSO was used as the vehicle control for NS-398 and GW9662. Hamilton syringe with a 27G needle was used for drug injection, which was given under the anesthesia. At the end of 24 h reperfusion, animals were sacrificed and brain infarct volume was determined by TTC staining. Rotarod test was also performed before and after the ischemic surgery to determine the motor function deficit of animals in each group. Overall, a total of 70 S.D. rats were included in the study, 7 of them were excluded from the study either due to the death caused by broken vessels (3 animals) or no brain infarction was induced (4 animals). The chloral hydrate/ketorolac-induced anesthesia or brain injection with NS-398 or GW9662 did not cause any fatal effect on ischemic rats.

TTC Staining

The infarct volume of ischemic rat was determined by TTC staining as previously described (Lin et al. 2016).

Rotarod Test

Rotarod test used in the study was based on our previous report (Lin et al. 2016). Sample size of each group is 3. Values in figure are the mean testing duration (min; how long an animal could hold on a rotating rod after ischemic surgery) of animals in each group divided by their mean internal baseline duration (min; how long an animal could hold on the rotating rod prior to ischemic surgery).

Double Immunofluorescence Staining

Brain expression of ADRP in microglia and TNF-α and IL-1β in brain adiposomes were determined by double immunofluorescence staining followed by the laser scanning confocal microscopy (LSCM) as previously described (Lin et al. 2016). The brain slices cut from ischemic hemisphere were 10 µm thin each and incubated with the primary antibodies for 15 h at 4 °C and followed by the secondary antibodies for 1 h at room temperature avoiding light. The primary antibodies used included the rabbit anti-ADRP (Santa Cruz; 1:50), goat anti-ADRP (Santa Cruz; 1:50), mouse anti-CD11b (GeneTex; 1:50), rabbit anti-TNF-α (Abcam; 1:100), and rabbit anti-IL-1β (Abcam; 1:200). The secondary antibodies used included the Alexa Fluor 594 (red)-conjugated goat anti-rabbit IgG (H + L), Alexa Fluor 488 (green)-conjugated rabbit anti-goat IgG (H + L), and Fluorescein isothiocyanate (green)-conjugated goat anti-mouse IgG (H + L), all from Jackson Immunoresearch (1:200). Microscopic observation for cells positive for ADRP/CD11b, ADRP/TNF-α, or ADRP/IL-1β expression was carried out with LSCM equipped with a 25-mW argon laser coupled to a fluorescence confocal microscope (Olympus Fv 1000). The excitation and emission wavelengths employed are 490 nm/525 nm for fluorescein (green), 560 nm/620 nm for rhodamine (red), and 430 nm/455 nm for DAPI (blue), respectively. The images were acquired in average accumulation mode using software FV10-asw 4.2 (14 scans in each sample). Percent (%) of the fluorescent stained cells/microscopic field in each group was determined by randomly selecting 10 microscopic fields from 2 brain slices per animal and then averaged the counts from 3 animals per group. Each number in the figures represents % of stained cells/field/animal/group ($n = 3$). Scale bar in the figures is 20 µm.

Statistical Analysis

Statistical analysis used in the study was Analysis of Variance (ANOVA) followed by multiple comparison test (Fisher PLSD), with α value of 0.05.

Results

Both Adiposomes and Inflammatory Activity of Microglia were Increased by GOSD

As mentioned early, microglia are pro-inflammatory cells and adiposomes are the reservoirs of inflammatory mediators, which can be found in macrophages and neutrophils. Could GOSD stimulate adiposome formation to potentiate the inflammatory activity of microglia was unclear and worthy to know. Figure 1 shows that in response to GOSD, number of adiposome (+) microglia determined by Oil Red O staining (Fig. 1a) was significantly increased by GOSD at 2, 4, and 6 h after treatment. The protein expression levels of inflammatory COX-2, TNF-α, and IL-1β in GOSD microglia were also determined and the level of COX-2 and TNF-α were both increased at 2 h and

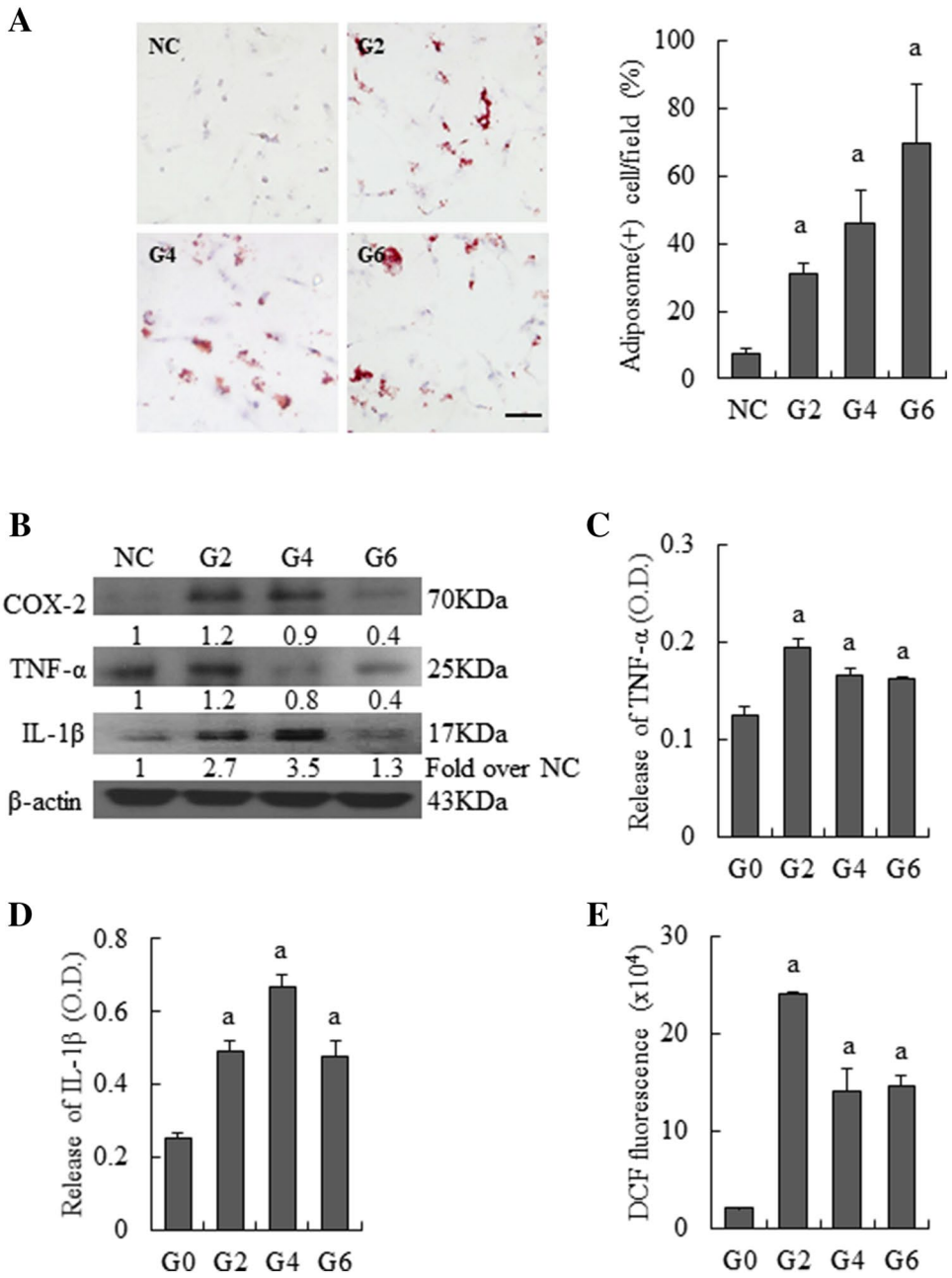

Fig. 1 GOSD can stimulate both adiposome formation and inflammatory activity of microglia. Microglia were subjected to GOSD for 0, 2, 4, or 6 h, followed by Oil Red O staining (**a**), Western blotting for COX-2, TNF-α, and IL-1β (**b**), ELISA for TNF-α (**c**) and IL-1β (**d**), and DCF assay for ROS release (**e**). *a* Significant difference between the indicated lanes and lane 1 (normal control group), $p < 0.05$. Each experiment was repeated 3 times. Scale Bar: 50 μm

then gradually declined at 4 and 6 h of GOSD treatment, whereas the level of IL-1β was increased at 2, 4, and 6 h of GOSD treatment and the maximal increase was at 4 h (Fig. 1b). Results from the ELISA and DCF assay further showed that the release of TNF-α (Fig. 1c), IL-1β (Fig. 1d), and ROS (Fig. 1e) from GOSD-treated microglia were all significantly increased at 2, 4, and 6 h of GOSD treatment. All these results demonstrated that GOSD was able to stimulate both adiposome formation and production of inflammatory mediators (TNF-α, IL-1β, and ROS) of microglia.

GOSD-Increased Adiposomes Played a Critical Role in Stimulating the Inflammatory Activity of Microglia, by Promoting the Production and Release of TNF-α and IL-1β

To clarify if GOSD-induced adiposome was responsible for the increased inflammatory activity of microglia, chemical blocking strategy was applied. Two inhibitors, NS-398 (adiposome and COX-2 inhibitor) and SC-58125 (COX-2 inhibitor), were used to distinguish the role of adiposome and COX-2 in the regulation of inflammatory activity of GOSD microglia. The results showed that GOSD

(2 h)-increased adiposome (+) microglia (Fig. 2a) were significantly blocked by NS-398 (at 10 and 100 μM) but not by SC-58125, both were given 2 h prior to GOSD. GOSD (2 h)-induced protein expression of COX-2 was blocked by NS-398 (at 10 and 100 μM) and SC-58125 (at 0.1, 1, and 10 μM), whereas the expression levels of TNF-α and IL-1β were only blocked by NS-398 (at 1, 10, and 100 μM) but not by SC-58125 (Fig. 2b). GOSD (2 h)-increased release of TNF-α (Fig. 2c) and IL-1β (Fig. 2d) were blocked only by NS-398 (at 1, 10, and 100 μM), whereas the release of ROS was blocked by both NS-398 (at 1, 10 and 100 μM) and SC-58125 (at 0.1, 1, and 10 μM) (Fig. 2e). These results demonstrated that NS-398 was able to block GOSD-increased adiposome formation and production of TNF-α and IL-1β, whereas SC-58125 was not. That could further prove GOSD-increased TNF-α and IL-1β were adiposome rather than COX-2 dependent, whereas GOSD-increased ROS was likely COX-2 rather than adiposome dependent. In other words, GOSD-increased adiposomes played a critical role in stimulating the inflammatory activity of microglia, by promoting the expression and release of TNF-α and IL-1β.

GOSD-Increased Adiposomes were Detrimental to Microglia and Their Surrounding Neighbor Neurons

The impact of GOSD-induced adiposomes upon the fate of microglia and their surrounding neighbor neurons was also investigated. Result from the trypan blue dye exclusion assay showed that GOSD (2 h)-decreased cell viability of microglia was significantly elevated by NS-398 pre-treatment (given 2 h ahead of GOSD; at 1 and 10 μM) (Fig. 3a), indicating GOSD-increased adiposomes were harmful to microglia under the GOSD condition. Figure 3b further shows that GOSD (6 h)-decreased cell viability of neurons was significantly elevated when GOSD neurons were cultured in 1 ml of MCM (microglia-derived conditioned medium collected at 2 h of GOSD treatment) (lane 3 versus lane 2, $p < 0.05$), indicating MCM could protect neurons from GOSD-induced cell death. Nevertheless, when GOSD (6 h)-treated neurons were co-cultured with MCM collected from GOSD (2 h)-treated microglia pre-treated with NS-398 (1 or 10 μM; 2 h prior to GOSD), cell viability of GOSD neurons was even further elevated (lanes 4 and 5 versus lane 3, $p < 0.05$). The results suggested that GOSD-increased adiposomes of microglia were also detrimental to the surrounding neurons, despite that the overall effect of MCM upon GOSD-treated neurons was protective (lanes 2 versus 3 in Fig. 3). The molecular mechanisms underlying MCM-mediated neuronal protection were discussed further in Discussion section. Overall, the results of Fig. 3a, b have demonstrated that GOSD-increased adiposomes of microglia were harmful not only to microglia but also their neighbor neurons.

PPARγ Could Partially Contribute to the GOSD-Increased Adiposome Formation to Potentiate the Inflammatory Activity and Cell Death of GOSD Microglia

PPARγ not just plays a dual role in inflammation, but its role in stimulating adiposome formation has also been reported in adipocytes and macrophages (Kim et al. 2016; Lin et al. 2017). Its roles in the regulation of adiposome formation and then inflammatory activity of GOSD microglia have never been reported before. Although microglia can express three different isoforms (PPARα, PPARβ/δ, and PPARγ) of PPARs (Kliewer et al. 1994), PPARα and PPARβ/δ have been reported to inhibit rather than stimulate the activity of microglia (Ramanan et al. 2009; Defaux et al. 2009). That has led us to temporarily preclude the possible involvements of PPARα and PPARβ/δ and focused mainly on the role of PPARγ in the regulation of adiposome formation and the inflammatory activity of GOSD-activated microglia. In the study, GW9662 (PPARγ inhibitor) or ROSI (PPARγ agonist) was used to clarify the role of PPARγ. Figure 4a shows that the GOSD-increased PPARγ expression could be seen throughout the first 6 h of GOSD treatment and the maximal increase was at 2 h of the treatment. Figure 4b shows that the GOSD (2 h)-increased PPARγ and ADRP expression were both blocked by GW9662 (1 and 10 μM; given 2 h prior to the GOSD treatment). Figure 4c shows that GOSD (2 h)-increased adiposome formation (Oil Red O staining) of microglia was also blocked by GW9662 (1 and 10 μM; 2 h prior to GOSD) but further increased by ROSI (10 μM; 2 h prior to GOSD). Furthermore, GOSD (2 h)-increased protein expression (but not release) of TNF-α and IL-1β (Fig. 4d) and cell death of microglia (Fig. 4e) were also blocked by GW9662 pre-treatment (1 μM). ROSI (1 and 10 μM; 2 h prior to GOSD), however, further exacerbated the GOSD-induced cell death of microglia (Fig. 4f). These results suggested that PPARγ played a critical role in the GOSD-increased adiposome formation, protein expression of TNF-α and IL-1β, and cell death of microglia. Nevertheless, PPARγ may only partially participate in the GOSD-increased adiposome formation because GOSD-increased release of TNF-α and IL-1β were only blocked by NS-398 (adiposome inhibitor) but not by GW9662 (PPARγ inhibitor). It is likely that besides PPARγ other signaling molecules (or pathways) may also contribute to the GOSD-increased adiposome formation and act together with PPARγ to promote full amount of adiposome production and lead to the release of inflammatory mediators (TNF-α and IL-1β).

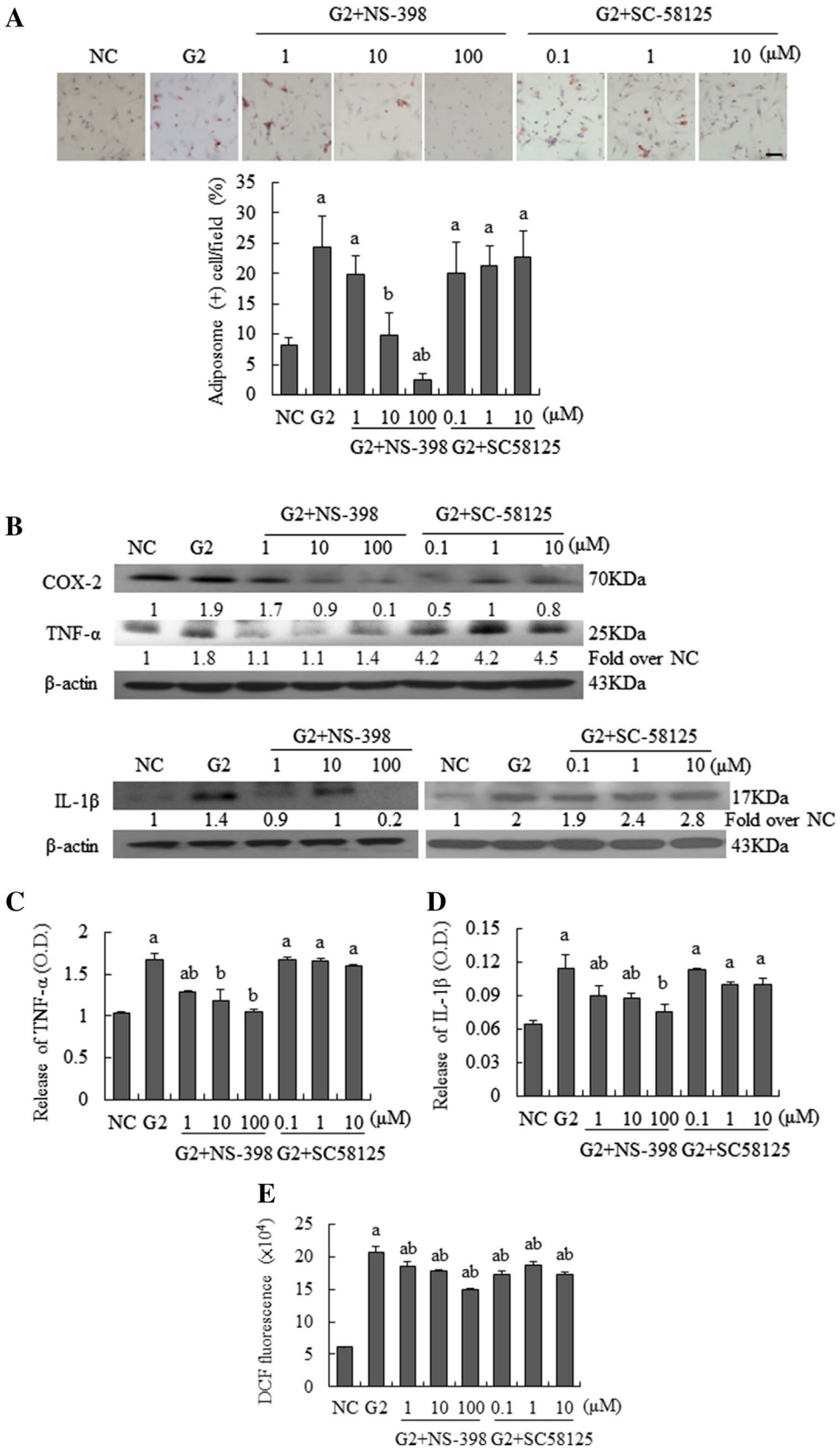

Fig. 2 Blockade of adiposome but not COX-2 could block GOSD-increased protein expression and release of TNF-α and IL-1β of microglia. Microglia were first treated with NS-398, SC-58125, or vehicle (0.1% DMSO) for 2 h and then followed by GOSD treatment for another 2 h. In the end of treatment, the Oil Red O staining for adiposome (+) microglia/field (**a**), Western blotting for COX-2, TNF-α and IL-1β expression (**b**), ELISA for TNF-α (**c**) and IL-1β (**d**) release, and DCF assay for ROS release (**e**), were all performed. *a* Significant difference between the indicated lanes and lane 1 (normal control group); *b* between the indicated lanes and lane 2 (GOSD only group), $p < 0.05$. Each experiment was repeated 3 times. Scale Bar: 50 μm

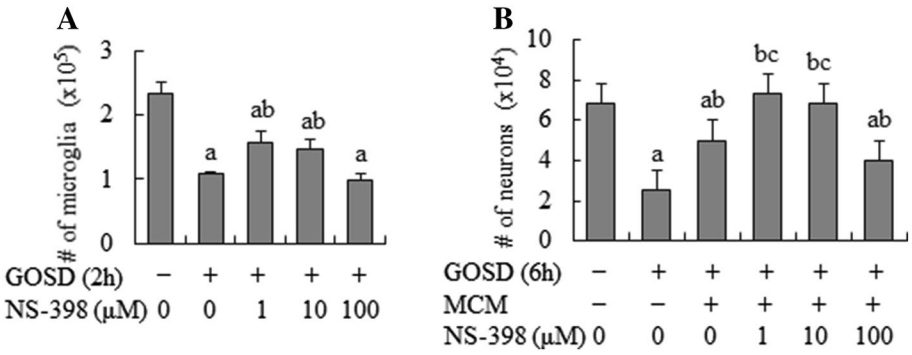

Fig. 3 GOSD-increased adiposomes could lead to death of microglia and their surrounding neighbor neurons. Microglia were pre-treated with NS-398 (1, 10, 100 μM) or vehicle (0.1% DMSO) for 2 h, followed by GOSD treatment for another 2 h. Cell viability of the treated microglia was determined by the trypan blue dye exclusion assay (**a**). MCM collected from GOSD (2 h) microglia, with or without the pre-treatment with NS-398 (1, 10, and 100 mM), was each added into neurons and co-cultured for 6 h, under the GOSD condition; cell viability of the treated neurons was then determined (**b**). *a* Significant difference between the indicated lanes and lane 1; *b* between the indicated lanes and lane 2; *c* between indicated lanes and lane 3, $p < 0.05$. Each experiment was repeated 3 times

Ischemia/Reperfusion-Induced Brain Infarction was Correlated with the Increase of Brain Adiposomes and the I/R-Increased Microglial Adiposomes May Serve as the Depots of TNF-α and IL-1β in Brain

The impacts of ischemia/reperfusion (I/R) upon the brain infarction, adiposome formation, and adiposome-associated expression of TNF-α and IL-1β were further examined in brain of S.D. rats. TTC staining showed that I/R-induced brain infarction was significantly increased at 12 and 24 h after reperfusion started (compared to sham control and I/R0h group, $p < 0.05$) and the maximal increase was seen at I/R24h (Fig. 5a). Oil Red O staining showed that I/R-induced adiposome formation was significantly increased at 0, 12, and 24 h after reperfusion started (compared to sham control, $p < 0.05$) and peaked at I/R24h (Fig. 5b). Double immunofluorescence staining followed by the laser scanning confocal microscopy was further used to know if I/R-increased adiposome formation was microglia associated. The results showed that adiposome-positive microglia (represented by % of ADRP (+)/CD11b (+) cells) in ischemic brain were significantly increased in I/R0h, I/R12h, and I/R24h group (compared to sham control group, $p < 0.05$) and degree of the increase remained relatively same in all I/R groups (Fig. 5c). I/R-induced inflammatory mediators were further co-localized with adiposomes (ADRP-positive) in ischemic brain. The results showed that percent of TNFα (+)/ADRP (+) cells and IL-1β (+)/ADRP (+) cells in ischemic brain were all significantly increased in I/R0h, I/R12h, and I/R24h group (compared to sham control group, $p < 0.05$) and no significant difference existed between all I/R groups (Fig. 5d, e). All these results suggested that I/R significantly increased the adiposome formation of microglia in ischemic brain. Furthermore, I/R-increased brain adiposomes may serve as the reservoirs of TNF-α and IL-1β that could further exacerbate the inflammation and infarction of ischemic brain.

NS-398 and GW9662 were Therapeutic Useful in Down-Regulating the I/R-Induced Brain Infarction and Motor Function Deficit

According to the pro-inflammatory and pro-death nature of adiposomes observed in GOSD microglia and ischemic brain, adiposome inhibition may have its potential in the control of cerebral ischemia. Therapeutic value of NS-398 and GW-9662 were, therefore, further examined in ischemic rats. Results from the TTC staining ($n = 3$ in each group) showed that I/R24h-induced brain infarction was significantly reduced when NS-398 (0.3 mg/kg) or GW9662 (0.1 mg/kg) was directly injected into the cisterna magna of ischemic brain, at 10 min prior to reperfusion started. Percent of the infarct volume in I/R + NS-398 and I/R + GW9662 group dropped from 26% (I/R + Vehicle control) to 11% and 15%, respectively, and there was no significant difference between the NS-398 and GW9662 group (Fig. 6a). The Rotarod test was also performed before and after I/R24h treatment and the results showed that I/R24h-induced motor function deficit was significantly improved by brain injection with NS-398 and GW9662 (the duration time holding on the Rotarod in I/R + NS398 and I/R + GW9662 group were all significantly longer than that of I/R group, $p < 0.05$) (Fig. 6b). All these results further supported the pathological role of adiposomes in cerebral ischemia and the therapeutic value of NS-398 and GW-9662 in the control of this disease.

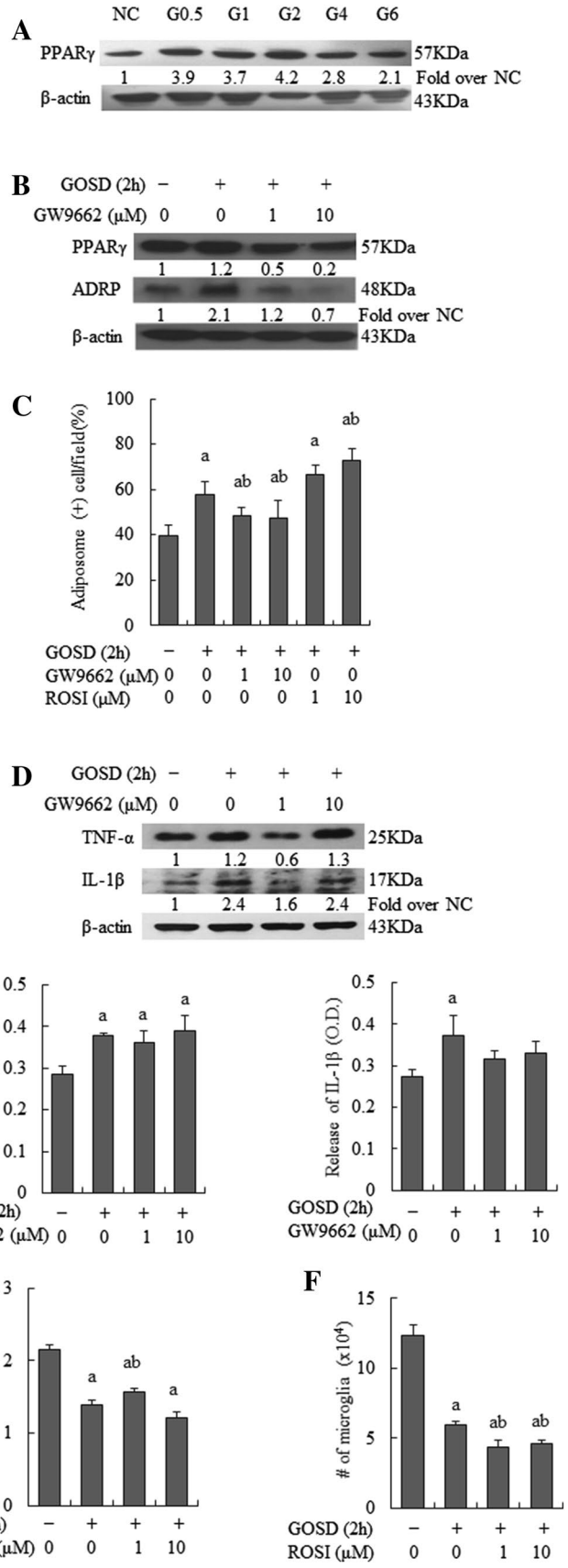

Fig. 4 GW9662 (PPARγ inhibitor) could block the GOSD-increased adiposome formation, protein expression of TNF-α and IL-1β, and cell death of microglia. Microglia were first incubated with GOSD for 0.5, 1, 2, 4, or 6 h to determine the expression levels of PPARγ by the Western blotting (**a**). In another set of experiments, microglia were pre-treated with GW9662 or ROSI for 2 h and then followed by GOSD treatment for another 2 h. The protein expression levels of PPARγ and ADRP were then determined by the Western blotting (**b**), percent of adiposome (+) cells/field by Oil Red O staining (**c**), the protein expression and release of TNF-α and IL-1β, by the Western blotting and ELISA (**d**), and survival of microglia by the trypan blue dye exclusion assay (**e, f**). *a* Significant difference between the indicated lanes and lane 1; *b* between indicated lanes and lane 2, $p < 0.05$. Each experiment was repeated 3 times

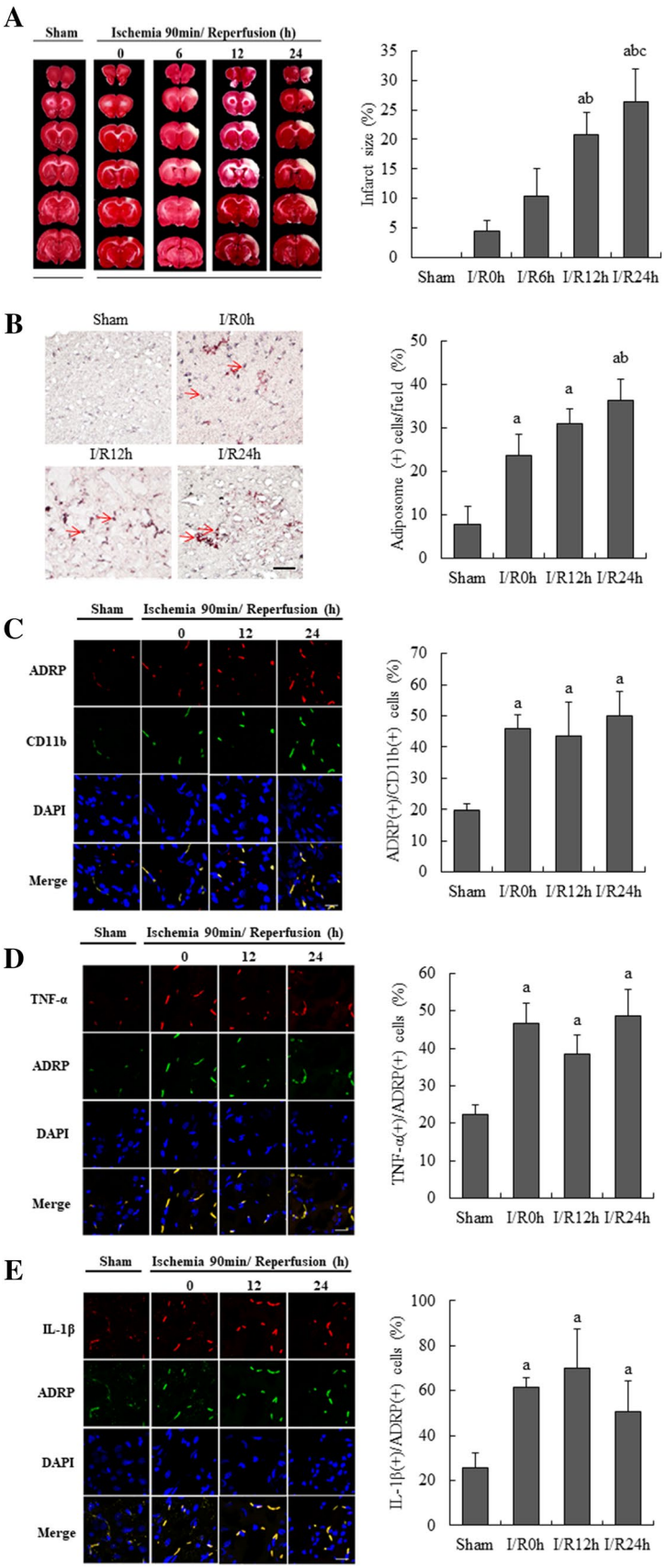

Fig. 5 Ischemia/reperfusion could significantly increase the brain infarct volume, formation of microglial adiposomes, and expression of adiposome-associated TNF-α and IL-1β in ischemic brain. S.D rats were subjected to MCAO/CCAO for 90 min and followed by reperfusion for various times as indicated. At each time points, the brain infarct volume was determined by TTC staining (**a**); percent of adiposome (+) cells/field by Oil Red O staining (**b**), percent of ADRP (+)/CD11b (+) cells per field (**c**), percent of TNF-α (+)/ADRP (+) cells per field (**d**), and percent of IL-1β (+)/ADRP (+) cells per field (**e**) were all determined by IHC staining followed by confocal microscopy. *a* Significant difference between the indicated lanes and lane 1; *b* between the indicated lanes and lane 2; *c* between indicated lanes and lane 3, $p < 0.05$. Sample size in **a** is 6 and in **b–e** 3 animals per group. Scale Bar: 50 μm in **b**; 20 μm in **c–e**

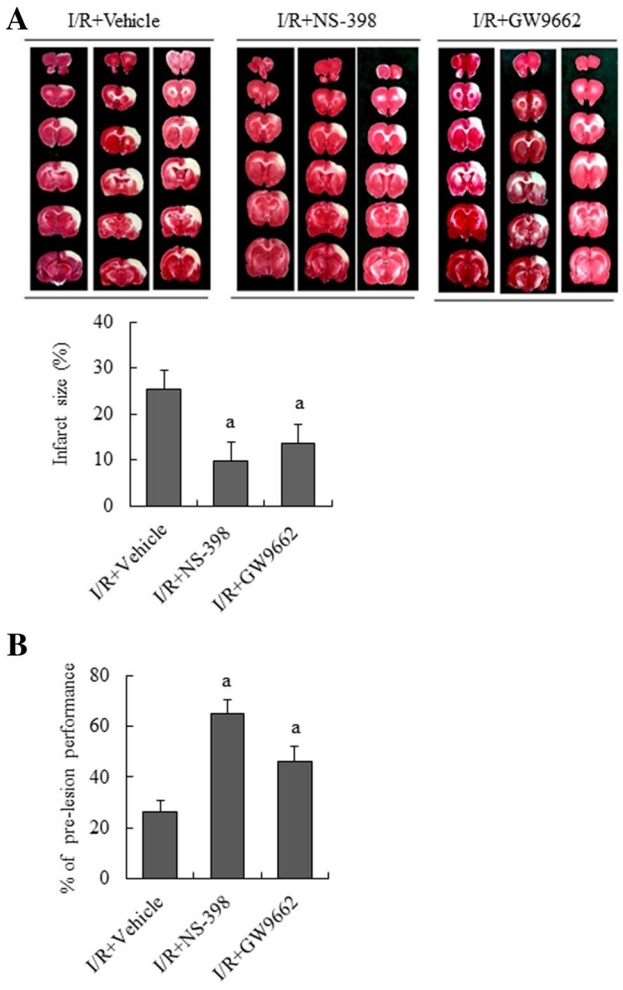

Fig. 6 Ischemia/reperfusion-induced brain infarction and motor function deficit were both blocked by NS-398 and GW9662. S.D rats were subjected to MCAO/CCAO for 90 min and followed by reperfusion for 24 h, in the absence or presence of NS-398 (0.3 mg/kg/15 µl) or GW9662 (0.3 mg/kg/15 µl). Each drug was injected via the cisterna magnum, 10 min prior to reperfusion started. DMSO (0.1%/rat/15µ l) was used as vehicle control. In the end of I/R24h surgery, animals were sacrificed and the mean brain infarct volume of each group was determined by TTC staining and averaged from 3 animals per group (**a**). Rotarod test was performed to determine the degree of motor function deficit of each group, based on the mean testing duration of animals in each group divided by their mean internal baseline duration (**b**). Experimental details please see the "Materials and Methods" section. *a* Significant difference between the indicated lanes and lane 1, $p < 0.05$. Sample size of each group is 3

Discussion and Conclusion

Ischemia-increased adiposome formation in brain has been reported before (Harada et al. 2007; Gasparovic et al. 2001) but its upstream regulator(s) and the pathological consequences were not clear. Here, through both GOSD and ischemic animal model, we have demonstrated that increase of adiposomes in ischemic microglia could promote the release of TNF-α and IL-1β to aggravate brain inflammation or even death of brain cells (microglia and neurons). The pro-inflammatory and pro-death nature of adiposomes has subjected these organelles to become potential therapeutic targets of stroke or other inflammatory diseases. NS-398 and GW-9662-mediated brain protection against cerebral ischemia further supported the notion. Adiposomes are known as the reservoirs of many inflammatory mediators, including COX-2, 5-LOX, TNF-α, IL-1β, PGE_2, LTB4 (leukotriene B4), and LTC4 (Beil et al. 1995; Bozza et al. 2002; Bandeira-Melo et al. 2001). The types of inflammatory mediators stored in adiposomes are, however, varied with cell type and stimulus. Our results for the first time showed that in response to ischemic insult, adiposomes of microglia could serve as the reservoirs of TNF-α and IL-1β (Figs. 2, 5) but not ROS or PGE2 (data not shown). However, it is worth to note that the pattern of adiposome formation was not quite the same as the production pattern of TNF-α and IL-1β in GOSD microglia (Fig. 1), indicating adiposomes may not be the only source (or regulator) of TNF-α and IL-1β in GOSD microglia.

The blocking effect of NS-398 and SC-58125 could help to distinguish the role of adiposome and COX-2 in the regulation of inflammatory responses of GOSD microglia. NS-398 is known to inhibit both COX-2 and ADRP synthesis (therefore adiposome formation) (Bozza et al. 2002, 1996), whereas SC-58125 only inhibits COX-2 synthesis. Our study showed that GOSD-increased TNF-α and IL-1β production in microglia were adiposome but not COX-2 dependent, whereas GOSD-increased ROS production in microglia was COX-2 but not adiposome dependent. Although adiposome-induced ROS production has been reported in macrophages, adipocytes, liver cells, and hepatoma (Lee et al. 2013), that was not the case in GOSD microglia. COX-2-stimulated ROS production was not only seen in our GOSD microglia but also in the injured hippocampal cells (Candelario-Jalil et al. 2000). Although COX-2 could stimulate TNF-α expression in hypoxic osteoblasts (Xing et al. 2015), and GOSD-induced COX-2 of microglia appeared to inhibit the expression of TNF-α and IL-1β (Fig. 2b), the different outcomes were likely due to cell type and stimulus difference. Additionally, COX-2 has been reported to promote adiposome formation in rat adipocytes, and lung and breast cancer cells (Wu et al. 2012; Goswami and Sharma-Walia 2016; Hung 2014), that obviously was not the case in GOSD microglia. GOSD-induced COX-2 showed no influence upon adiposome formation of GOSD microglia (Fig. 2a, b). The discrepancy again may result from cell type and/or stimulus variation. COX-2-independent adiposome formation has also been reported in cancer cells (Assumpcao et al. 2017) and non-cancer cells such as adipocytes and macrophage (Lin et al. 2017).

The impact of microglia-derived adiposomes upon survival of microglia and their neighbor neurons were also investigated under the GOSD condition and proven to be detrimental to both. Microglia-derived adiposomes have revealed a pro-death effect upon GOSD microglia and GOSD neurons based on the facts that adiposome deprivation by NS-398 significantly increased the survival of GOSD microglia and further elevated the MCM-mediated protection of GOSD neurons (Fig. 3a, b). We have previously demonstrated that MCM (microglia-derived conditioned medium collected under the GOSD condition) could protect brain cells (microglia, astrocytes, and neurons) from GOSD-induced cell injury, in a TGF-β1-, GDNF-, or NT-3-dependent manner (Lee et al. 2004; Lu et al. 2005). The results of our cytokine antibody array study, however, have shown that MCM actually contained a variety of molecules released from GOSD microglia (un-shown data) (Lin et al. 2016), some of these molecules were beneficial but others may be detrimental to microglia and/or their neighbor cells under the ischemic stress. The results of Fig. 3b have newly demonstrated that GOSD-induced adiposomes of microglia could lessen the overall protective effect of MCM, likely by releasing more inflammatory molecules (such as TNF-α or IL-1β) into MCM (Figs. 1, 2). Adiposome-released TNF-α and IL-1β could aggravate inflammation but may also contribute to the adiposome-mediated cell death of GOSD microglia and neurons (Fig. 3) because TNF-α- and IL-1β-triggered cell death have been reported before (Hundsberger et al. 2008; England et al. 2014). Collectively, despite the energy compensatory effect (pro-survival) of adiposomes, GOSD-increased adiposomes of microglia appeared to be detrimental based on their pro-inflammatory and pro-death effect. That further explained why overproduction of adiposomes could be considered as the therapeutic targets in the treatment of cerebral ischemia (stroke) and NS-398 was therapeutic useful in the control of this disease.

PPARγ is capable of regulating adiposome formation and inflammation. It can stimulate the expression of ADRP and then adiposome formation by activating the PLD-1/ERK/Dynein signaling pathway (Andersson et al. 2006; Gao et al. 2010). PPARγ-increased adiposome formation has been reported in cancer cells (Assumpcao et al. 2017) and non-cancer cells such as adipocytes and macrophages (Kim et al. 2016; Lin et al. 2017). Our results newly demonstrated that PPARγ could also increase adiposome formation in GOSD microglia to aggravate the inflammatory activity and death rate of the cells. PPARγ also dually regulates inflammation. Most of the reports suggest that PPARγ is an anti-inflammatory mediator and inhibits NF-κb activity to down-regulate the expression of TNF-α, IL-1β, and other inflammatory mediators (Duan et al. 2008). Our results, however, have shown that GOSD-induced PPARγ promoted the expression of TNF-α and IL-1β to boost the inflammatory activity of GOSD microglia. The pro-inflammatory effect of PPARγ has also been reported in the Tityus serrulatus-activated macrophages (Zoccal et al. 2015). It is worth to note that PPARγ may only partially contribute to the GOSD-induced adiposome formation and the subsequent inflammatory responses of GOSD microglia because GW9662 only blocked the GOSD-increased protein expression but not release of TNF-α and IL-1β, whereas NS-398 can block both events in GOSD microglia. In other words, PPARγ has to work with other signaling molecules (or pathways) to fully complete the GOSD-increased adiposome formation, leading to sufficient amount of TNF-α and IL-1β release from GOSD microglia.

Adiposome formation of microglia and adiposome-associated TNF-α and IL-1β expression have also been examined in ischemic brain and appeared to be correlated with I/R-induced brain infarction. Therapeutic values of NS-398 and GW9662 in the control of cerebral ischemia have also been confirmed. Although PPARγ may only partially contribute to the GOSD-induced adiposome formation and inflammatory activity of microglia, GW9662-mediated brain protection was as good as NS-398-mediated protection, indicating PPARγ may act through multiple ways (not just adiposome stimulation) to exacerbate I/R-induced brain infarction. On contrary to our findings, PPARγ agonist (TZD) had also been reported to protect brain from ischemic infarction (Tureyen et al. 2007; Fong et al. 2010). The discrepancy has warned us the complex roles of PPARγ in cerebral ischemia or other inflammatory diseases and how it works still requires further investigation. Previously, NS-398 had been reported to protect mouse brain from cerebral ischemia by acting as a COX-2 inhibitor (Nagayama et al. 1999). Our study has provided new insights about the protective mechanisms of NS-398 in the control of cerebral ischemia, by revealing its role in adiposome inhibition.

In conclusion, the study has newly demonstrated the pro-inflammatory and pro-death role of adiposome in GOSD microglia and ischemic brain. PPARγ in part contributed to the GOSD-increased adiposome formation of microglia. Adiposome blockade with NS-398 or GW9662 appeared to be therapeutic useful in the control of cerebral ischemia, likely via the inhibition of inflammatory activity and cell death of microglia.

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Author Contributions CHL analyzed the data and wrote the paper; LYL designed and executed most of the experiments and analyzed the data; YJC performed the experiments in Figs. 4d and 2d; CWT performed the experiments in Figs. 4e and 5d; TYY and SLH supported the work

and edited the paper; CMH conceived the project, designed experiments, supervised others, and edited the manuscript.

Compliance with Ethical Standards

Conflict of interest The authors have no financial and non-financial conflicts of interest.

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