Adiponectin ameliorates GMH-induced brain injury by regulating microglia M1/M2 polarization via AdipoR1/APPL1/AMPK/PPAR γ signaling pathway in neonatal rats

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Abstract

Adiponectin (APN), a fat-derived plasma hormone, is a classic anti-inflammatory agent. Multiple studies have demonstrated the beneficial role of APN in acute brain injury, but the effect of APN in Germinal matrix hemorrhage (GMH) is unclear, and the underlying molecular mechanisms remain largely undefined. In the current study, we used a GMH rat model with rh-APN treatment, and we observed that APN demonstrated a protective effect on neurological function and an inhibitory effect on neuroinflammation after GMH. To further explore the underlying mechanisms of these effects, we found that the expression of Adiponectin receptor 1 (AdipoR1) primarily colocalized with microglia and neurons in brain. Moreover, AdipoR1, but not AdipoR2, were largely increased in GMH rats. Meanwhile, further investigation showed that APN treatment promoted AdipoR1/APPL1-mediated AMPK phosphorylation, and further increased peroxisome proliferator-activated receptor gamma (PPAR γ) expression and induced microglial M2 polarization to reduce neuroinflammation and enhance hematoma resolution in GMH rats. Importantly, either knock down of AdipoR1, APPL1, or LKB1, or specific inhibition of AMPK/ PPAR γ signaling in microglia abrogated the protective effect of APN after GMH in rats. In all, we propose that APN works as a potential therapeutic agent to ameliorate the inflammatory response following GMH by enhancing the M2 polarization of microglia via AdipoR1/APPL1/AMPK/PPAR γ signaling pathway, ultimately attenuating inflammatory brain injury induced by hemorrhage.

Introduction

Germinal matrix hemorrhage (GMH) is the most common type of intracranial hemorrhage to occur in preterm neonates and is caused by the rupture of blood vessels in the periventricular subependymal immature region (i.e., germinal matrix) [1]. Destruction of the precursor cells within the germinal matrix and post-hemorrhagic hydrocephalus in infants suffering from GMH [2] may lead to development of significant long-term neurocognitive sequelae, including mental retardation, seizures, and cerebral palsy [3]. Unfortunately, no therapies have been shown to be effective in treating neonatal GMH, and the only preventive intervention is perinatal glucocorticoids [4]. As the rates of survival in preterm births and neonates have substantially increased in recent decades, GMH-induced neurological deficits are increasingly becoming a socio-economic burden. Therefore, safe, and effective pharmacologic treatments are desperately needed.

The initial pathological damage of cerebral hemorrhage is the mechanical compression to adjacent tissues caused by hematoma [5]. Secondary damage is known to be triggered by blood clots that contribute to neuroinflammation and neurological dysfunction, including post-hemorrhagic hydrocephalus [6], reactive astrogliosis [7] and microgliosis [8]. Secondary brain injury plays critical roles in neurological deterioration outcomes in hemorrhagic stroke including intracerebral hemorrhage, subarachnoid hemorrhage and GMH. As therapeutic developments against primary injury have demonstrated definitive benefits in clinical trials, investigators have instead focused on exploring the mechanisms that underlie post-GMH secondary brain injury in the pursuit of novel targets for treatment.

Increasing evidence suggests that inflammatory reactions play a key role in the pathogenesis of stroke and contribute to secondary brain injury after hemorrhage [9, 10]. Microglia/macrophages are the major immune cells of the central nervous system (CNS) and are critical drivers of the neuroinflammatory response after various of hemorrhagic brain injuries [11]. Upon activation, microglia develop into either classically activated (M1) or alternatively activated (M2) phenotypes, a process termed polarization. M1 microglia are initially present following an insult as they promote an inflammatory response. Conversely, M2 microglia mainly secrete anti-inflammatory cytokines and growth factors, and may facilitate neuronal recovery, hematoma resolution, and vascular remodeling. Additionally, in response to distinct microenvironmental cues, an already fully polarized M1 or M2 sub-population can reverse its phenotype and function [12, 13]. Therefore, strategies aiming to promote the phenotypic conversion of microglia from M1 to M2 might provide therapeutic potential for GMH.

Adiponectin (APN) is a plasma hormone predominantly secreted by adipocytes, and is known to exert powerful anti-apoptosis/anti-inflammatory effects in acute and chronic brain injury, including ischemic or hemorrhagic stroke, mainly through the Adiponectin receptor 1 (AdipoR1) and AMP-activated protein kinase (AMPK) pathway [14]. We previously found that adiponectin could exert neuroprotective effects by attenuating neuronal apoptosis via AdipoR1/AMPK signaling pathway after neonatal rats are subjected to hypoxic ischemic injury [15]. Recently, emerging evidence has shown adiponectin signaling pathway has a detrimental role in regulating the immune response via microglia from the central nervous system (CNS) [16, 17]. Furthermore, AMPK signaling pathway activation could accelerate hematoma resolution and improve neurological outcomes after intracerebral hemorrhage (ICH) [18].

Thus, in this present study, we hypothesize that APN will attenuate neuroinflammation and accelerate hematoma clearance through the AdipoR1/AMPK signaling pathway after GMH by regulating the M1/M2 polarization.

Methods

Animals

Two hundred and fifty-five Postnatal day (P) 7 Sprague-Dawley neonatal pups (weight = 12-15 g, Harlan, Livermore, CA) of either sex were randomly assigned into the either Sham (n=56) or GMH surgery (n=199) groups. Of the 199 rats in the GMH surgery group, no pups were excluded from the study as a result of intraoperative death. All pups were kept in rooms with controlled temperature and ad libitum to drink and chow. The design of experiments, timeline, and animal numbers per group are depicted in Supplementary Fig. 1.

Germinal matrix hemorrhage (GMH) induction

GMH induction was conducted in unsexed P7 rats using collagenase infusion as previously described [19, 20]. Briefly, rat pups weighting between 12-15g were anesthetized using 3% isoflurane, and anesthesia was maintained using 1.5% isoflurane intraoperatively. The heads were fixed in place on a stereotaxic frame,

and the scalp was then sterilized. An incision was made to expose the bregma, and a burr hole (1mm) was drilled at 1.6 mm right lateral and 1.5 mm rostral relative to bregma. A 10 μ L Hamilton syringe (Hamilton Co, Reno, NV, USA) filled with 0.3 U/ μ L collagenase solution was fixed to an infusion pump (Harvard Apparatus, Holliston, MA) and the needle was inserted 2.8 mm below the dura with the bevel facing the midline. Next, 0.3 U type VII-S collagenase from *Clostridium histolyticum* (Sigma Aldrich St. Louis, MO) was infused at a rate of 0.3 U/3min, and the needle remained in place for 5 min before being removed at a rate of 1 mm/min to prevent possible leakage. The burr hole was sealed with bone wax, and the incision was sutured with 5-0 silk. Animals were allowed to recover on a 37°C-heating pad then returned to the dam after awakening from anesthesia. The sham surgery followed the same procedure as described above, but without collagenase infusion. The average duration of surgery for each animal was approximately 30 min.

Drug administration

Recombinant human adiponectin protein (rh-APN) (Pepro Tech, NJ, USA) was administered by intranasally at 1h post-GMH in three different doses (0.05 mg/kg, 0.1 mg/kg, and 0.3 mg/kg) as described previously [15]. The rh-APN was administered intranasally once daily for 3 days post-GMH (short-term study) or 7 days with best dosage (long-term study).

In vivo RNAi

As previously described [15, 19], 2 μ l rat derived AdipoR1 siRNA (300 pmol/ μ l, Life Technologies), APPL1 siRNA (300 pmol/ μ l, Life Technologies), LKB1 siRNA (300 pmol/ μ l, Life Technologies) or Scramble siRNA were delivered via intracerebroventricular injection at 24 h prior to GMH induction (1.5 mm posterior, 1.5 mm lateral to the bregma and 1.7 mm deep from the surface).

Administration of Liposomes

Liposomes (FormuMax Scientific, CA, USA) that contain a lipid fluorescent dye, Lipo-DHPE (Fluorescein DHPE), Lipo-Dorsomorphin (Dorsomorphin, AMPK inhibitor, Santa Cruz Biotechnology, USA) or Lipo-GW9662 (GW9662, PPAR γ inhibitor, Sigma Aldrich, MO, USA) were prepared according to manufacturer's protocol as previously reported [19]. The final concentration of liposomal Dorsomorphin (6 μ g/ μ l) and GW9662 (12 μ g/ μ l) were determined by microplate reader system (400nm, SpectraMax i3x, Molecular Devices, Thermo Fisher Scientific, USA).

Immunofluorescence staining

Double Immunofluorescence staining was performed on fixed frozen brain sections as described previously [19]. The 8- μ m thickness slides were rinsed with phosphate-buffered saline (PBS) and permeabilized with 0.3% Triton X-100 for 15min at RT, followed with blocking solution (95% PBS, 5% normal donkey serum, and 0.05% Triton X-100) for 2 hours. Slides were then incubated with mouse anti-Iba1 (1:200, Abcam, Cambridge, MA, USA), rabbit anti-APN (1:50, Abcam, Cambridge, MA, USA), rabbit anti-AdipoR1 (1:100, Abcam, Cambridge, MA, USA), goat anti-NeuN (1:200, Abcam, Cambridge, MA, USA), rabbit anti-IL6 (1:100, Abcam, Cambridge, MA, USA), rabbit anti-CD68 (1:100, Abcam, Cambridge, MA, USA)

or rabbit anti-CD206 (1:100, Abcam, Cambridge, MA, USA) at 4°C overnight. After washing with PBS for three times (10min each time), the sections were then incubated with the appropriate fluorescent secondary antibodies (diluted 1:200) (Jackson Immuno Research) and counterstained with DAPI (Vector Laboratories). All counts and quantifications were performed in a blinded fashion as previously described [19, 21].

Western blot analysis

Brain tissues were homogenized in RIPA lysis buffer (Santa Cruz Biotechnology, USA) for at least 60s. The homogenate was centrifuged at 15,000 rpm/min at 4°C for 20 min and the supernatant collected, aliquoted, and then stored at -80°C. Protein concentrations were determined by via DC protein assay (Bio-Rad, USA). Western blots were processed as previously described [15]. Briefly, 30µg of protein per sample were loaded into wells of 10% gels, ran for 30 min at 80 V, and then for 90 min at 120 V. Proteins were transferred onto 0.2µm or 0.4µm nitrocellulose membrane at 100 V for 120 min (Bio-Rad, USA). The following primary antibodies were applied to the membranes and incubated overnight at 4°C: anti-Adiponectin, anti-AdipoR1, anti-AdipoR2, anti-APPL1, anti-LKB1, anti-p-AMPK, anti-AMPK, anti-PPAR γ , anti-CD36, anti-CD68, anti-CD206, anti-IL-6, anti-IL-1 β , anti-TNF α (1:1000; Abcam, USA) and Actin (1:1000; Santa Cruz Biotechnology, USA). Membranes were washed and incubated in appropriate secondary antibodies (1:2000; Santa Cruz Biotechnology, USA) for 2h at RT. Fiji software (NIH Images, USA) was used to analyze the relative density as described [21].

Hemoglobin assay

Spectrophotometric measurement to assess hemorrhagic volume was conducted as previously described [21, 22]. Frozen extracted forebrain tissues were placed into individual glass test tubes containing 3 mL of PBS, followed by homogenization for 60s with the help of Tissue Miser Homogenizer (Thermo Fisher Scientific, USA). After ultrasonication for 1 min to lyse the erythrocyte membranes, the products were then centrifuged for 30 min and the supernatant was separated from the pellets. Then, Drabkin's reagent was added (Sigma-Aldrich, USA) into aliquots of supernatant in a ratio of 4:1, which were allowed to react for 15 min. Finally, Absorbance was calculated into a hemorrhagic volume (µL) on the basis of a standard curve, as routinely performed using a spectrophotometer (540; Genesis 10uv; Thermo Fisher Scientific, USA) [21].

Neurological tests

Neurobehavioral function was evaluated blindly in a random and unbiased setup as previously reported [23, 24]. Short-term neurological tests, such as righting reflex and negative geotaxis tests, were performed on days 1 to 3 post-GMH. Long-term neurological tests, including water maze, rotarod, and foot-fault, were performed on days 21-28 post-GMH.

Righting reflex test

The time for each pup to completely rollover onto from supine to prone was recorded. The maximum recoding time was 60s (3 trials/pup/day). The average values of all three trials were calculated.

Negative geotaxis test

Pups were placed head downward onto a 45° inclined plane, and the time to rotate 180° was recorded. The maximum duration was 20 s (3 trials/pup/day). The average value of all three trials were calculated.

Water maze test

Morris water maze testing was conducted to assess animal memory and learning capacity as previously described. The apparatus consisted of a metal pool (110 cm in diameter) accompanied by a small platform (11 cm in diameter), in which the pups to climb on. Swim distance, latency, and velocity were digitally recorded and analyzed using a tracking software (Noldus EthoVision, USA). In the cued test, pups were manually guided to the platform if they had difficulty in locating it, and the location of the platform was changed every other trial. In hidden tests, the platform was submerged 1 cm below the water, and the time spent in probe quadrant was recorded.

Quantitative real-time PCR (qRT-PCR) analysis

To determine the level of gene expression, total RNA was isolated from brain tissue using RNeasy kit (Qiagen) and cDNA were synthesized using GoScript Reverse Transcriptase (Promega) according to the manufacturer’s instructions and as previously described [19]. The RNA expression was measured by qRT-PCR with specific primers (Table 1) using Bio-Rad iQ5 system (Hercules).

Table 1
Primers used in real-time PCR for the target genes.

Gene Name	GenBank Number	Sense (5'-3')	Anti-sense (5'-3')
Adiponectin	NM_144744.3	TGTTCTTGGTCCTAAGGGTGAC	CCTACGCTGAATGCTGAGTGA
AdipoR1	NM_207587.2	CAGAAAACCCAGCAGTTGCC	AAAGGAAACACCCACTGCCA
AdipoR2	NM_001037979.1	GGAGTGTTCGTGGGCTTGGG	GCAGCTCCTGTGATATAGAGG
Arginase-1	NM_017134.3	AAGACAGGGCTACTTTCAGGA	CAAGACAAGGTCAACGCCAC
GADPH	NM_001106123.2	GGTTCC GGTTTGTGGAGCAG	TCCGTTTGCATTGCCCAGTA

Nissl staining

On day 28 after GMH, the whole brains were collected and fixed in 10% formalin in preparation for histology after euthanasia. The brains were sliced into 16-18µm thick slices (Leica Microsystems, LM3050S) after being embedded with Optimal Cutting Temperature (OCT, Fisher Scientific, Waltham, MA). Then the coronal brain slices were stained with 0.5% Cresyl violet and imaged using a microscope (Olympus-BX51) [19]. Ventricular volume, white matter loss and cortical thickness were evaluated as reported previously [6, 19].

Statistical analysis

All data are presented as mean $\pm$ SD. Statistical analysis was performed using SPSS v.24.0 (IBM Corp., USA). Statistical differences were performed using One-way ANOVA with Dunnet's post-hoc test for multiple comparison and two-tailed Student's t test for two group comparisons. $P \leq 0.05$ was considered statistically significant.

Results

Time course and spatial expression of endogenous Adiponectin, AdipoR1 and AdipoR2 after GMH

Endogenous Adiponectin, AdipoR1 and AdipoR2 expression were assessed by western blot analysis at 0h (Sham), 12h, 24h, 72h, and 7d in the ipsilateral hemisphere after GMH. The results showed that the expression of endogenous APN (Fig. 1a, b) and AdipoR1 (Fig. 1a, c) were significantly increased at 12h, peaked at 72h, and decreased at 7d after GMH when compared to Sham group. However, no changes were observed in expression of AdipoR2 after GMH (Fig. 1a, d). Consistently, the expression pattern of Adiponectin, AdipoR1 and AdipoR2 were identified by qRT-PCR analysis (Supplementary Fig. 2b-d). In addition, double immunofluorescence staining showed that Adiponectin and AdipoR1 were abundantly expressed in microglia, neurons (Fig. 1e, f), and astrocytes (Supplementary Fig. 2a) surrounding the lateral ventricle of pups with GMH but were barely expressed in microglia and neurons (Fig. 1e, f) found around the periventricular area of pups that were not subjected to GMH.

Intranasal administration of rh-APN ameliorated neurological deficits and attenuated inflammation in 72h after GMH

To investigate the translational treatment regimen of APN, three doses (0.05mg/kg, 0.1mg/kg, 0.3mg/kg) were administered in a single treatment intranasally 1h post-GMH. More time cost on flipping to the prone position and rotating to head upward position in vehicle-treated groups when compared to the Sham group at 1d and 2d post-GMH (Fig. 2a, b). Improved short-term neurological function was observed in all three doses of rh-APN treated pups. Interestingly, the medium dose of rh-APN-treated pups displayed the best performance in both righting reflex (Fig. 2a) and negative geotaxis tests (Fig. 2b), which was compared to sham group as early as at 2d after GMH. Collectively, 0.1mg/kg of rh-APN treatment was used for all of the following experiments.

Anti-human Adiponectin antibody was used to evaluate the level of exogenous rh-APN in the brain after intranasal treatment. As shown in Fig. 2c, more rh-APN were observed in brain tissue when compared to vehicle treated group. A key feature of stroke involves the secretion of inflammatory cytokines [25]. The anti-inflammatory effects of APN have been previously reported in intracerebral hemorrhage (ICH) rat model [26]. Herein, the protein expression levels of pro-inflammatory (IL-1 β and IL-6) and anti-inflammatory cytokines (IL-10) in perihematomal brain tissue were detected. The expression of pro-inflammatory cytokines dramatically increased, and anti-inflammatory cytokine expression levels were

slightly increased 72h after GMH (Fig. 2c). However, with the treatment of rh-APN, the dramatic increase of pro-inflammatory cytokines (IL-1 β and IL-6) were blunted, which was further validated by immunostaining of IL-6 in the choroid plexus (Fig. 2d). Simultaneously, anti-inflammatory cytokines, IL-10 (Fig. 2c) and Arginase-1 mRNA expression (Fig. 5h) by qRT-PCR were significantly promoted.

Rh-APN treatment improved long-term neurological function at 21-28 days after GMH

To investigate the effects of rh-APN treatment on the long-term neurological impairments induced by GMH, neurological functions were assessed by water maze, foot-fault, and rotarod tests at 4 weeks post-GMH. In the Morris Water Maze evaluation, Vehicle-treated rats significantly swam longer (Fig. 3a), spent more time seeking the platform (Fig. 3b) and spent less time in the defined quadrant zone (Fig. 3c, d) when compared to the sham group. Meanwhile, rh-APN-treated rats had a significantly greater performance than vehicle-treated rats as demonstrated by shorter travel distance, decreased time to escape, and more time spent in the target quadrant zone (Fig. 3a-d). However, there is no significant difference in swimming velocity among these three groups (Fig. 3e). Rats in the vehicle groups have a significantly shorter latency to fall at both of 5RPM and 10RPM acceleration compared to the sham group in the rotarod test. Rh-APN treatment significantly reduced the falling latency compared to the vehicle, However, there were no significant differences when compared with sham (Fig. 3f). Additionally, rh-APN treatment also significantly improved sensorimotor function after GMH in the foot fault test compared to the vehicle group (Fig. 3g). Regarding the growth profile, there was significantly slowed normal growth from 14d to 28d after GMH was observed in vehicle-treated animals compared to sham group, but rh-APN restored normal body weight (Fig. 3h).

GMH-induced microglial M1 phenotype polarization was prevented by rh-APN via AdipoR1 signaling pathway

Alternative polarization of microglia exerts a beneficial role in attenuating inflammation after brain hemorrhage including GMH [20, 27, 28]. To explore whether the microglia M1/M2 polarization involved in attenuating inflammation mediated by rh-APN treatment after GMH, the M1 phenotype marker (CD68) and M2 phenotype marker (CD206) were examined by immunostaining assay. Data showed that the percentage of CD68 and Iba1 double-positive cells increased at 72 h after GMH, but significantly decreased following rh-APN administration (Fig. 4a, b). Simultaneously, rh-APN treatment significantly increased the percentage of CD206 and Iba1 double-positive cells after GMH when compared with the vehicle group (Fig. 4a, c). Importantly, the ratio of M1-like (CD68⁺Iba1⁺) cells/M2-like (CD206⁺Iba1⁺) cells decreased with administration of rh-APN (Supplementary Fig. 3e). However, the rh-APN-mediated effects in M1/M2 polarization, as described above, were reversed by knockdown of AdipoR1 with si-AdipoR1 (Fig. 4a-c). In sham animals, microglia remained in their ramified state with or without rh-APN treatment (Fig. 4a-c).

Consistent with the immunofluorescence staining results, western blot analysis showed that the expression of M1 marker, CD68, was significantly increased and M2 marker D206 expression was slightly

increased at 72 h post-GMH. However, administration of rh-APN significantly decreased CD68 expression and promoted CD206 expression, and the effects were significantly reversed by si-AdipoR1 (Fig. 4d), indicating that rh-APN promoted M2 polarization while inhibiting M1 polarization via AdipoR1.

rh-APN treatment promoted accumulation of alternatively activated M2 microglia in the periventricular area after GMH

Microglia undergo morphological, phenotypic and functional changes following GMH induction. These changes include an increase in number through proliferation, a shift from ramified to amoeboid morphology, and secretion of chemokines. To explore whether treatment with rh-APN affected the microglial response after GMH, pan microglia marker (Iba1), and activated microglia/macrophage marker (CD11b/c+) were detected in the periventricular area. Iba1 positive-stained microglia in both vehicle and rh-APN-treated groups showed significant morphological changes when compared to sham group at 72 h after GMH (Fig. 5a).

Given that microglia proliferation has protective effects after hemorrhage, Ki67⁺/Iba1⁺ microglia, as well as BrdU⁺/Iba1⁺ microglia was evaluated in the periventricular area at 72 h post-GMH. As shown in Fig. 5a-d, the number of Ki67⁺/Iba1⁺ and BrdU⁺/Iba1⁺ microglia were significantly increased in rh-APN- and vehicle-treated pups compared to sham. However, there is no significant difference between rh-APN and vehicle groups with a slightly increase (Fig. 5b, d). Interestingly, the number of activated microglia (CD11b/c⁺) was significantly increased in the periventricular area in rh-APN group when compared with the vehicle group (Fig. 5e, f).

rh-APN administration enhanced hematoma resolution and reduced ventricular dilation by increasing M2 microglia in the periventricular area after GMH

Peroxisome proliferator-activated receptor gamma (PPAR γ) is a pivotal transcription factor and plays a major role in upregulating CD36 expression [29]. Multiple studies have shown that PPAR γ plays a major role in promoting polarization of microglia to the M2 phenotype in various experimental stroke models [30, 31]. Hence, expression of PPAR γ and CD36 expression were evaluated by western blot analysis. The results showed that administration of exogenous rh-APN further augmented PPAR γ and CD36 expression at 72h post-GMH (Fig. 4e, f).

Since a previous study demonstrated PPAR γ -induced upregulation of CD36 could enhance hematoma resolution and ameliorate secondary brain injury after GMH [21], hemoglobin assay time-course was conducted at 24 hours, 72 hours, and 7 days post GMH to further explore the effects of rh-APN treatment on hematoma clearances. At 24 hours after GMH, all groups had significantly greater hemoglobin content in the ipsilateral brain compared to sham, but there were no significant differences between GMH groups either with/without treatment of rh-APN or si-AdipoR1 (Fig. 6b). However, rh-APN treatment had

significantly reduced hemoglobin volume compared to vehicle controls at 72 hours post-GMH, which was reversed by AdipoR1 knockdown with si-AdipoR1 RNA (Fig. 6a, c). Additionally, the vehicle-and rh-APN-treated groups had greater hemoglobin content compared to sham at 7 days after GMH, but the rh-APN-treated group had significantly less hemoglobin content compared to the vehicle group (Fig. 6d).

Given that alternatively activated microglia (M2 phenotype) play a pivotal role in hematoma clearance [5, 21], we evaluated CD206+ microglia in the periventricular area at 72 h after GMH. No CD206+ microglia were observed in the sham group, while vehicle-treated pups showed a slight increase in the number of CD206+ microglia. Importantly, with the knockdown of AdipoR1 by si-AdipoR1 RNA, we observed fewer CD206-positive microglia in si-AdipoR1 group when compared to vehicle groups (Fig. 6e, f). By contrast, treatment of rh-APN significantly increased CD206+ microglia compared to vehicle, whereas this increase was abrogated by knockdown of AdipoR1 (Fig. 6e, f).

Furthermore, double immunostaining showed that CD206 was co-located with hemoglobin (marker of hematoma) in the periventricular area in pups after GMH with rh-APN treatment, indicating rh-APN promoted hematoma resolution by CD206+ microglia mediated phagocytosis (Fig. 6g).

Additionally, to evaluate whether rh-APN alleviated the severity of ventriculomegaly, the ventricular volume was assessed at 28 days post-GMH. As shown in Supplementary Fig. 3a, significant ventricular dilation occurred in vehicle group when compared to sham. However, rh-APN treatment significantly reduced the ventricular volume when compared to vehicle-treated pups (Supplementary Fig. 3a, b). White matter loss was markedly increased in the vehicle group, but it was significantly restored with administration of rh-APN (Supplementary Fig. 3a, d). Concurrently, the cortical thickness was markedly decreased in the vehicle-treated pups, whereas rh-APN treatment significantly reduced the cortical loss (Supplementary Fig. 3a, d).

rh-APN attenuates neuroinflammation and promotes hematoma resolution via AdipoR1/APPL1/LKB1/AMPK signaling pathway at 72h post-GMH

To investigate whether AdipoR1/APPL1/LKB1/AMPK signaling protect neonatal brain against hemorrhage after GMH, the specific AdipoR1 siRNA, APPL1 siRNA, LKB1 siRNA and their negative control (scramble siRNA) were intracerebroventricularly injected 24 hours before GMH induction. Western blot data showed that AdipoR1, APPL1, p-AMPK, PPAR γ , CD36, CD206 and IL-10 expression significantly increased in the vehicle group when compared with sham group (Fig. 7a-c, e-h, k). Administration of rh-APN further upregulated their expression compared to the vehicle-treated group. In contrast, the M1 marker CD68 (Fig. 7a, i) and proinflammatory cytokine IL-1 β (Fig. 7a, g) expression was dramatically increased, whereas rh-APN treatment decreased their expression when compared with the vehicle group. Knockdown of AdipoR1 with AdipoR1 siRNA (si-AdipoR1) significantly decreased AdipoR1 expression and abolished the effects of rh-APN (Fig. 7a-k). As shown in Fig. 7a, silencing of AdipoR1 expression was associated with decreased APPL1, AMPK phosphorylation, PPAR γ , CD36, CD206 and IL-10 expression, but was associated with increased CD68 and IL-1 β expression after GMH with rh-APN treatment. Furthermore, knockdown of

APPL1 and LKB1 expression showed similarly trends as knockdown of AdipoR1 was associated with decreased AMPK phosphorylation and PPAR γ , CD36, CD206 and IL-10 expression (Fig. 7a, f-h, k), but with increased CD68 and IL-1 β expression (Fig. 7a, i, j).

Selective inhibition of AMPK/PPAR γ signaling in activated microglia cells abolished the effect of rh-APN on inhibiting neuroinflammation and promoting hematoma resolution

To directly test whether microglia AMPK/PPAR γ signaling mediated a protective effect of rh-APN post-GMH, we delivered specific AMPK/PPAR γ inhibitors into microglia cells via intracerebroventricular injection of Lipo-Dorsomorphin (1 μ g/g, 2 μ l) and Lipo-GW9662 (2 μ g/g, 2 μ l) 24 h before GMH was induced as described in our previous study [19]. Green fluorescence-labeled liposomes (Fig. 8a) were mainly found in activated IBA+ microglia at 72 h post GMH (Fig. 8a), whereas no labeled liposomes were observed in Neurons (Red, NeuN+) and Astrocytes (Red, GFAP+) (Fig. 8a).

The addition of Lipo-Dorsomorphin and Lipo-GW9662 to rh-APN did not affect AdipoR1 expression (Fig. 8b, c). The phosphorylation of AMPK (Fig. 8b, d) and PPAR γ (Fig. 8b, e) expression were significantly reduced in Lipo-Dorsomorphin-treated pups compared to rh-APN with or without Lipo-PBS-treated pups. In addition, Lipo-GW9662 inhibited PPAR γ expression, but had no effect on AMPK phosphorylation (Fig. 8b, e, f), indicating that PPAR γ is a downstream kinase of AMPK after GMH. Administration of the two liposome-encapsulated inhibitors significantly increased CD68 expression but decreased CD36 and CD206 expression when compared with rh-APN or rh-APN + Lipo-PBS treated pups (Fig. 8b, f-h). Collectively, AMPK/PPAR γ inhibition in activated microglial cells restored the inflammation after GMH, indicating a key role of microglial AMPK/ PPAR γ signaling in protecting the neonatal brain from hemorrhage-induced damage.

Discussion

Germinal matrix hemorrhage (GMH) is the most commonly occurring neurological disorder associated with premature newborns, with an incidence of 3.5 per 1,000 live births [32] and is defined as blood vessel rupture of subependymal immature near the ganglionic eminence [33]. Herein, we explored the protective effects of APN on post-GMH brain injury and shed light on mechanisms that could be involved. We found that rh-APN could improve short-term and long-term neurobehavioral outcomes by attenuating GMH-induced inflammation. Mechanistically, rh-APN decreased proinflammatory cytokines and increased anti-inflammatory cytokine secretion by preventing GMH-induced microglial polarization to the M1 phenotype while promoting the acquisition of the M2 phenotype. Furthermore, rh-APN enhanced hematoma resolution by promoting accumulation of M2 microglia with enhanced phagocytic ability in the periventricular regions. Finally, Western blot data showed that knockdown of AdipoR1, APPL1 and LKB1, as well as selectively inhibited AMPK phosphorylation and PPAR γ in activated microglia reversed APN's effects. Taken together, in the present study, we found that APN facilitated the M1 to M2 microglial

phenotypic conversion after GMH. AdipoR1-mediated activation of the APPL1/LKB1/AMPK/PPAR γ pathway played a crucial role in the M1 to M2 transformational process, as well as process of hematoma resolution (Fig. 9).

Increasing evidence indicates that pronounced inflammation plays critical roles in GMH-induced secondary brain injury [27, 34, 35]. Therefore, we evaluated inflammatory-related cytokines after GMH. Hemorrhagic brain injury in GMH causes a dramatic increase in production of pro-inflammatory cytokines (IL-1 β and IL-6) within the brain, which can subsequently lead to hydrocephalus. The inflammatory response following GMH aggravated GMH-induced hydrocephalus, ultimately leading to tissue damage, blood-brain barrier (BBB) disruption, and massive brain cell death. Following hemorrhagic stroke, pro-inflammatory molecules are released from the damaged neurons or astrocytes, resulting in microglial activation, and further increasing the release of inflammatory cytokines [36, 37]. The ability of microglia to acquire diverse states of activation reflects different features that are determinant of their activity in these key processes of GMH recovery, such as neuroinflammation, tissue repair, or immunomodulation. Classically activated (M1, CD68+) microglia reportedly release destructive pro-inflammatory mediators, whereas alternatively activated (M2, CD206+) microglia to clear tissue debris through phagocytosis and release numerous protective and trophic factors. M1 microglia polarization mainly act in the acute phase after GMH, resulting in an overwhelming release of pro-inflammatory cytokines [27]. Consistently, we found that microglia primarily polarized to the M1 phenotype with few M2 phenotype microglia at 72 hours after GMH.

Microglia/macrophages are characterized by remarkable plasticity and versatility, such as being able to switch from one phenotype to another. In mice with collagenase-induced ICH, the M1 to M2 microglial phenotype switch was observed from days 1 to 3 after ICH [38]. Stimulation of cannabinoid receptor 2 (CB2R) suppressed neuroinflammation by regulating M1/M2 polarization via cAMP/PKA signaling pathway in an experimental GMH rat model [27]. Recently, we reported that GW9508-mediated GPR40 activation attenuates neuroinflammation and improves neurological function through PAK4/CREB/KDM6B signaling pathway after GMH [20]. In the current study, our data showed that intranasal administration of rh-APN promoted M2 microglia polarization with increased secretion of anti-inflammatory cytokines (IL-10 and Arginase-1).

In addition, we explored the effects of adiponectin on sham animals, and the results showed that adiponectin did not influence microglial polarization in the absence of GMH. Combined with IHC staining, it was shown that rh-APN did not influence microglial activation in sham animals, and we demonstrated that rh-APN drives microglial M2 polarization on the basis of an activated state. Moreover, we found that knockdown of AdipoR1 reversed the effect of rh-APN and increased expression of AdipoR1 on alternatively activating (M2, CD206-positive) microglia after rh-APN treatment. Taken together, it is extremely likely that the necessary transition from the M1-activated state to the M2 phenotype using rh-APN relies on upregulation of AdipoR1 expression.

Microglial proliferation and accumulation play detrimental roles in the pathology of GMH in the immature preterm brain [39]. Tang et al. reported that activation of CB2R inhibited thrombin-induced microglial

proliferation and the inflammatory response. We found increased microglia proliferation following GMH and rh-APN treatment had no effect on the proliferation of GMH. However, increased accumulation of activated microglia or macrophages (CD11b/c+) in the periventricular area were observed after GMH with rh-APN treatment. Meanwhile, administration of rh-APN significantly increased M2 (CD206⁺) microglia, which indicates that the activated and accumulated microglia in the periventricular region might be predominantly M2 (CD206⁺) microglia.

Peroxisome proliferator-activated receptor- γ (PPAR γ) is a ligand-activated transcription factor belonging to the nuclear hormone receptor superfamily. Activation of PPAR γ mediated the conversion of microglia phenotype and phagocytic capabilities of peripheral M2 polarized macrophages in various CNS diseases [40, 41], including hemorrhagic stroke [21]. Flores et al. demonstrated that the PPAR γ -induced upregulation of CD36 in microglia increased M2 microglia polarization and was critical in enhancing post-GMH hematoma resolution.

PPAR γ agonists enhanced myelination, reduced inflammation and hydrocephalus, and promoted neurological recovery in newborns with intraventricular hemorrhage (IVH) [42]. More recently, studies have shown that activation of adiponectin receptor with AdipoRon could boost PPAR γ expression and inhibit pro-inflammatory microglia responses, therefore ameliorates hyperperfused cognitive deficits [41]. Accordingly, we demonstrate that APN promote phenotypic change of microglia from “classically” M1 activated to alternatively activated M2 states and enhances hematoma clearance, thereby reducing hydrocephalus by activation of PPAR γ via AdipoR1 signaling. Emerging evidence has shown that the activation of AMPK promoted microglial M2 polarization, thereby inhibiting neuroinflammation after stroke [43–45]. Moreover, APN attenuated neuroinflammation after intracerebral hemorrhage through the AdipoR1-AMPK pathway [46]. Since APPL1 and LKB1 were involved in APN/AdipoR1-mediated AMPK activation [15], AMPK might participate in APN-induced upregulation of PPAR γ . Intraventricular administration of specific siRNA-targeted APPL1 and LKB1 significantly reversed AMPK activation and increased PPAR γ expression induced by rh-APN/AdipoR1. In addition, CD36 and IL-10 expression were decreased, while IL-1 β and IL-6 expression were increased after knockdown of APPL1 and LKB1 was followed with APN treatment, suggesting that APN alleviated GMH-induced inflammation by upregulation of PPAR γ via AdipoR1/APPL1/LKB1/AMPK signaling.

Considering that the expression of AdipoR1 can be found on various brain cells and the role of AdipoR1/AMPK/PPAR γ signaling in microglia in neuroinflammation in vivo remains unexplored, we used the Lipo-Dorsomorphin (AMPK inhibitor) and Lipo-GW9662 (PPAR γ antagonist) to inhibit AMPK and PPAR γ activation selectively in microglia, as previously described [19]. With the inhibition of AMPK activation and decreased PPAR γ expression, the CD36 and IL-10 expressions were completely reversed with rh-APN treatment. By contrast, expression of IL-1 β and IL-6 were significantly increased, suggesting that rh-APN attenuated neuroinflammation may be primarily mediated through the AMPK/PPAR γ pathway in microglia. However, in the present study, we mainly focused on the effects of APN, which were mediated by microglial signaling post-GMH. The effects of APN on neurons or astrocytes will be further investigated.

Conclusion

In conclusion, the present study highlights the neuroprotective effect of APN in reducing proinflammatory cytokine release and in enhancing hematoma resolution by promoting M2 microglial polarization and accumulation of M2 microglia in the periventricular area, thereby alleviating neurological deficits in an experimental GMH rat model. The neuroprotective effects of APN were associated with the AdipoR1/APPL1/AMPK/PPAR γ signaling pathway.

Abbreviations

APN: Adiponectin; AdipoR1: Adiponectin receptor 1; AdipoR2: Adiponectin receptor 2; GMH: Germinal matrix hemorrhage; APPL1: Adaptor Protein, Phosphotyrosine interacting with PH domain and Leucine Zipper 1; LKB1: Liver kinase B1; AMPK: AMP-activated protein kinase; Peroxisome proliferator-activated receptor gamma; CNS: Central nervous system; CD68: Cluster of Differentiation 68; CD206: Cluster of Differentiation 206; ICH: Intracerebral hemorrhage; RPM: Revolutions per minute; SD: Standard deviation; ANOVA: One-way analysis of variance.

Declarations

Ethics approval and consent to participate

All experimental procedures were conducted in compliance with the National Institutes of Health guidelines and were approved by the Loma Linda University Institutional Animal Care and Use Committee.

Consent for publication

Not applicable.

Availability of data and material

Information about the experimental methods, animal model, and the datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests with respect to the research, authorship, and/or publication of this article.

Authors' contributions

NBX, XFL, DMD, JPT, JHZ and CZD conceived the research idea and experimental design. NBX, XFL, JW, CHW, ZYH, WHT, CL, JZ, YCL, QX and YHL performed experiments and analyzed the data. NBX, XFL, JW, CL and XYH drafted the manuscript. Critical revisions of the manuscript were made by all authors. CZD approved the final version of the manuscript on behalf of all the authors.

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Figures

Figure 1

Endogenous Adiponectin and AdipoR1 were upregulated in the brain after GMH. (A-C) Western blot data showed that the endogenous Adiponectin (A, B) and AdipoR1 (A, C) expression levels significantly increased from 12h to 3d and peaked at 3d post-GMH. (A, D) AdipoR2 expression levels were comparable between pups with or without GMH at four time points. Values are expressed as normalized to Actin protein expression. Values are expressed as mean $\pm$ SD. ANOVA, Dunnett. * $P < 0.05$ compared to sham, $n = 6$ for each group. (E) Representative images of immunofluorescence staining showing the co-localization of Adiponectin and AdipoR1 (red) with microglia and neurons (Iba1, NeuN, green) in the pups with or without GMH. Adiponectin and AdipoR1 immunoreactivities were greater on microglia and neurons in the periventricular area at 72h after GMH. Arrows indicated Adiponectin or AdipoR1 colocalized with microglia. Scale bar = 50 μ m. $n = 3$ for each group.

Figure 2

Intranasal administration of Adiponectin improved short-term neurological function at 72h post-GMH. (A) Righting reflex and (B) Geotaxis reflex tests showed that medium dose (0.1mg/kg) of rh-APN significantly improved neurological function compared to vehicle treated pups at 1d, 2d and 3d after GMH. Values are expressed as mean $\pm$ SD. ANOVA, Dunnett. $n = 6-9$ for each group. * $P < 0.05$ compared to sham, # $P < 0.05$ compared to GMH + Vehicle. (C) Western blot data showed that intranasal administration of rh-APN after GMH could be delivered successfully into brain tissue, and it reduced proinflammatory cytokine levels of IL-1 β and IL-6 while increasing expression levels of anti-inflammatory cytokine, IL-10, within the brain. ANOVA, Dunnett. $n = 6$ for each group. * $P < 0.05$ compared to sham, # $P < 0.05$ compared to GMH + Vehicle. (D) Immunofluorescence staining assay showed IL-6 immunoreactivity in the choroid plexus was dramatically increased in pups subjected to GMH relative to sham, and rh-APN treatment significantly reduced the IL-6 immunoreactivity compared to GMH.

Figure 3

rh-APN administration improved long-term memory and motor function at 21-28 days after GMH. Water maze test (A-E) showed that rh-APN treatment significantly improved spatial learning and memory performance with reduced swim distance to find the platform (A), less time to escape (B), and more time spent in the probe quadrant (C-D). However, no significant difference in swim velocity was found among the three groups (E). rh-APN treatment notably improved motor function of pups assessed by rotarod (F)

and foot fault (G) tests after GMH. (H) Histograms showing the weight changes of pups with or without rh-APN treatment in 4 weeks post-GMH. Values are expressed as mean $\pm$ SD. ANOVA, Dunnett. $n = 8$ for each group. * $P < 0.05$ compared to sham, # $P < 0.05$ compared to GMH + Vehicle.

Figure 4

rh-APN treatment promoted M2 microglia polarization at 72h post GMH. (A) Representative images of immunofluorescence staining and quantification (B-C) showing the co-localization of CD68 (M1 marker, red) or CD206 (M2 marker, red) and Iba-1 (green). Scale bar = 50 μ m. (D) Representative image of western blot data showing the expression of CD68, CD206, PPAR γ and CD36. (E-H) Western blot quantification showed that the expression of CD68 was markedly increased and CD206, PPAR γ and CD36 expression

were slightly increased compared to sham after GMH. (D-H) rh-APN significantly decreased CD68 expression, while increased the expression of CD206, PPAR γ and CD36. (D-H) However, these effects were reversed by knockdown of AdipoR1 with AdipoR1 siRNA. Values are expressed as mean $\pm$ SD. ANOVA, Dunnett. $n = 6$ for each group. * $P < 0.05$ compared to sham, # $P < 0.05$ compared to GMH + Vehicle, @ $P < 0.05$ compared to GMH + rh-APN or GMH + rh-APN + Scramble siRNA.

Figure 5

rh-APN promoted accumulation of M2 microglia in the periventricular area 72 h after GMH. (A, C) Representative images of immunofluorescence staining and quantification (B, D) showed accumulation and proliferation (Ki67+/BrdU+, red) of microglia (Iba+, green) in the periventricular region. (E-F) Representative images of immunofluorescence staining and quantification showing activated microglia/macrophages (CD11b/c+, green) in the periventricular region. Scale bar = 50µm. Dots in A-G represent data from individual pups. FOV=2.3 $\times$ 10⁶ µm³. Values are expressed as mean $\pm$ SD. ANOVA,

Dunnett. $n = 6$ for each group. $^*P < 0.05$ compared to sham, $^{\#}P < 0.05$ compared to GMH + Vehicle. (G) Representative images of immunofluorescence staining showing co-localization of AdipoR1 (red) and M2 microglia (CD206+, green) in the periventricular regions after GMH. Scale bar = 50 μ m. $n = 3$ for each group. Arrows indicate AdipoR1 colocalized with M2 microglia. (H) qRT-PCR assay showed that rh-APN significantly increased the mRNA expression of Arginase-1 at 72 h post-GMH. Values are expressed as mean $\pm$ SD. ANOVA, Dunnett. $n = 6$ for each group. $^*P < 0.05$ compared to sham, $^{\#}P < 0.05$ compared to GMH + Vehicle.

Figure 6

rh-APN promoted hematoma clearance by increasing M2 microglia in the periventricular area after GMH. (A) Representative images of hematoma in the periventricular area of brain at 72 h after GMH. (B-D) Hemoglobin assays were conducted at (B) 24 hours, (C) 72 hours, and (D) 7 days. (E) Representative images of immunofluorescence staining and (F) quantification showing the accumulation of M2 microglia in the periventricular regions after GMH. Scale bar = 50 μm. n = 6 for each group. Values are expressed as

mean $\pm$ SD. ANOVA, Dunnett. $n = 6$ for each group. * $P < 0.05$ compared to sham, # $P < 0.05$ compared to GMH + Vehicle, @ $P < 0.05$ compared to GMH + si-AdipoR1, & $P < 0.05$ compared to GMH + rh-APN or GMH + rh-APN + Scramble siRNA. (G) Representative images of immunofluorescence staining showing co-localization of CD206 with hemoglobin in periventricular area after GMH with rh-APN treatment. Scale bar = 50 μ m, Upper panel; Scale bar = 50 μ m, lower panel.

Figure 7

AdipoR1/APPL1/LKB1/AMPK signaling is a potential pathway for rh-APN afforded anti-inflammation in GMH pups. (A) Representative images of western blot data showing the expression of AdipoR1, APPL1, LKB1, p-AMPK, PPAR γ , CD36, CD68, CD206, IL-1 β , and IL-10 either with rh-APN treatment alone, rh-APN + si-AdipoR1, rh-APN + si-APPL1 or rh-APN + si-LKB1. (B) Western blot analysis of AdipoR1 showed that AdipoR1 levels increased in rh-APN treatment group and decreased in the si-AdipoR1 group, while AdipoR1 expression does not change in si-APPL1 or si-LKB1 pups with rh-APN treatment. (C) Western blot analysis

of APPL1 showed that APPL1 expression was increased in the rh-APN-treated group and decreased in si-AdipoR1 and si-APPL1 groups. However, there were no significant changes of APPL1 levels after knockdown of LKB1 with si-LKB1. (D) Western blot analysis of LKB1 showed that LKB1 levels decreased in si-LKB1 treated group. (E) Western blot analysis of p-AMPK to AMPK ratio showed that p-AMPK increased in the rh-APN-treated group and decreased in si-AdipoR1, si-APPL1, and si-LKB1 groups. (F, G, I, K) Western blot data showed that (F) PPAR γ , (G) CD36, (I) CD206, and (K) IL-10 expression increased in rh-APN treated group and decreased in si-AdipoR1, si-APPL1 and si-LKB1 groups. (H, J) Western blot data showed that (H) CD68 and (J) IL-1 β expression were significantly decreased with rh-APN treatment while si-AdipoR1, si-APPL1, and si-LKB1 reversed the inhibitory effects of rh-APN. Values are expressed as mean $\pm$ SD. ANOVA, Dunnett. n = 6 for each group. * P < 0.05 compared to sham, # P < 0.05 compared to GMH + Vehicle, @ P < 0.05 compared to GMH + rh-APN or GMH + rh-APN + Scramble siRNA.

Figure 8

Selective inhibition of AMPK/PPAR γ signaling in activated microglia cells abolished the effect of rh-APN on inhibiting neuroinflammation and promoting hematoma resolution 72 hours after GMH (A)

Representative images of immunofluorescence staining showing fluorescent dye-labeled liposomes (a, green) were swallowed almost entirely in microglia (d, e) (Iba1 $^{+}$, red) rather than in Neurons (b) (NeuN $^{+}$, red) and Astrocytes (c) (GFAP $^{+}$, red) at 72h after GMH. (B) Representative images of western blot data showing the expression of p-AMPK, PPAR γ and CD36, as well as CD68 and CD206 either with rh-APN treatment alone, rh-APN + Lipo-Dorsomorphin or rh-APN + Lipo-GW9662. (C) No changes observed in the expression of AdipoR1 with Lipo-Dorsomorphin and Lipo-GW9662 intervention. (D) Western blot analysis

of p-AMPK to AMPK ratio showed p-AMPK increased in the rh-APN treatment group and decreased in Lipo-Dorsomorphin group. (E, F, H) Western blot data showed (E) PPAR γ , (F) CD36 and (H) CD206 expression increased with rh-APN treatment but decreased in Lipo-Dorsomorphin and Lipo-GW9662 groups. (G) Western blot data showed that rh-APN significantly decreased CD68 expression, whereas Lipo-Dorsomorphin and Lipo-GW9662 reversed the inhibitory effect of rh-APN. (All samples of GMH + rh-APN in western blot were from the same animals which were euthanized after short-term neurobehavioral tests). Values are expressed as mean $\pm$ SD. ANOVA, Dunnett. n = 6 for each group. * P < 0.05 compared to sham, # P < 0.05 compared to GMH + Vehicle, @ P < 0.05 compared to GMH + rh-APN or GMH + rh-APN + Lipo-PBS.

Figure 9

The potential molecular mechanisms of APN-mediated brain protection exerted their effect by enhancing microglial M2 polarization, attenuating neuroinflammation and promoting hematoma resolution via AdipoR1/APPL1/AMPK/PPAR γ signaling after GMH.

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