Apolipoprotein M in High Density Lipoprotein Protects Against Astrocyte Apoptosis Induced By Ischemic Insult Via Sphingosine 1-Phosphate Signaling

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Abstract

Apolipoprotein M (ApoM) has been reported to be contained in high density lipoprotein (HDL) and bound with sphingosine-1-phosphate (S1P). ApoM-associated HDL exerts protective effects against cell death. The aims of the study were to evaluate the effects of ApoM-associated HDL on astrocytes following ischemic insult. Primary cultured mouse astrocytes were treated with oxygen-glucose deprivation (OGD) followed by recovery. The astrocytes underwent apoptosis after treatment with OGD for 4 h and recovery for 24 h. The addition of HDL with ApoM attenuated apoptotic cell death, but HDL without ApoM did not show any effect. Free S1P or ApoM-bound S1P promoted cell survival and inhibited apoptosis. Only S1P receptor 1 (S1PR1) expression was upregulated and blockage of S1PR1 with specific inhibitor or genetic knockdown of *S1pr1* abolished the protective effects. In addition, administration of ApoM containing HDL or free S1P induced activation of Akt and Erk in the astrocytes, and pharmacological inhibition of Akt and Erk rescued cell death after OGD treatment. Taken together, ApoM is required for the protective effects of HDL, which depends on delivery of S1P to S1PR1 by ApoM in HDL, indicating ApoM may be a neuroprotective constituent in plasma.

1. Introduction

As the most abundant cell type in the brain, astrocytes are important for the maintenance of cerebral function in physiological conditions and homeostasis of blood-brain barrier. During cerebral ischemia and reoxygenation, neurons may undergo cell death resulting from accumulation of free oxygen radicals (Tajiri, Oyadomari et al. 2004, Peng, Zhao et al. 2015). Astrocytes can produce free radical scavenger substances and participate in the protection of neurons against oxidative injury (Papadopoulos, Koumenis et al. 1997, Murthy, Rao et al. 2001, Fulmer, VonDran et al. 2014). On the other hand, astrocytes can function as the defense of neurons by providing trophic support and physical barrier of glial scar (Huang, Li et al. 2014), preventing massive neuronal death. Astrocytes are also vulnerable to ischemic insults, thus, protection of astrocytes against cell death may be essential to prevent neuronal injuries as well as to maintain normal brain function. So far, little is known about the endogenous factors in blood that regulate glial cell survival.

Recently studies suggest that high density lipoproteins (HDL) in blood exhibits therapeutic efficiency for the treatment of cardiovascular disease due to the effects of healthy HDL in vitro (Luscher, Landmesser et al. 2014, Ruiz, Okada et al. 2017), indicating the novel function of HDL. Among the apolipoproteins in HDL, apolipoprotein M (ApoM) plays an important role in the protection. ApoM belongs to the lipocalin family and functions as a lipid carrier in blood (Liu, Allegood et al. 2015). The gene expression of ApoM is restricted to liver and kidneys in human and ApoM protein is predominantly enriched in HDL (Xu and Dahlback 1999, Kurano, Hara et al. 2014). Based on its chemical structure, ApoM possesses an eight-stranded β -barrel with a hydrophobic binding pocket, suggesting its binding affinity with some hydrophobic substances in plasma (Mathiesen Janiurek, Soyulu-Kucharz et al. 2019). It has been reported that different ligands such as oxidized phospholipids and sphingosine 1-phosphate (S1P), can bind with apolipoproteins in vivo (Kurano, Hara et al. 2014). Among these bioactive lipids, only S1P is the

physiological ligand for ApoM in vivo and most of S1P is carried by ApoM in blood (Liu, Allegood et al. 2015, Dusaban, Chun et al. 2017). Approximate 5% of HDL particles in blood carry ApoM-S1P complexes and are involved in lipid metabolism (Blaho, Galvani et al. 2015). Recently, some evidences show that ApoM participates in the homeostasis maintenance of endothelial monolayer (Zhu, Luo et al. 2018, Mathiesen Janiurek, Soyly-Kucharz et al. 2019). ApoM knockout mice exhibit reduced HDL-associated S1P in blood and increased vascular permeability (Zhu, Luo et al. 2018, Mathiesen Janiurek, Soyly-Kucharz et al. 2019), indicating the role of ApoM/S1P axis in the vascular function.

S1P is a kind of sphingolipids and possesses several key physiological functions, including regulation of cell growth and survival. S1P exerts its effects via binding and activating its receptors, that is S1P receptors (S1PR, S1PR1-5), then activates specific downstream signals (Van Doorn, Van Horssen et al. 2010, Cartier, Leigh et al. 2020). The most investigated S1PR is S1PR1, which is involved in cell trafficking, angiogenesis and cell survival (Ruiz, Okada et al. 2017, Mathiesen Janiurek, Soyly-Kucharz et al. 2019, Cartier, Leigh et al. 2020). Modulation of S1PR1 with chemical compound has shown efficiency to treat some disorders. For example, one of the S1PR1 modulators, FTY720 prevents lymphocyte egress from lymphoid organs and receives approval as an oral treatment for relapsing forms of multiple sclerosis (Kappos, Antel et al. 2006). Thus, S1PR1 may be a useful therapeutic target for metabolic diseases.

Recently, it has been proved that HDL particles protect against cell apoptosis in endothelial cells, which is related to the increased cholesterol efflux mediated by ApoM (Luscher, Landmesser et al. 2014, Galvani, Sanson et al. 2015). The goal of this study is to characterize the participation of ApoM and S1P in the regulation of astrocyte apoptosis and the signaling pathways involved. In the study, we investigated the effects of HDL particles on primary cultured mouse astrocytes. We demonstrated that ApoM-containing HDL attenuated astrocyte apoptosis induced by ischemic insult and recovery in vitro. The protective effects are mediated by ApoM-S1P complex and require the activation of Akt or extracellular-signal-regulated kinase (Erk) pathways. The results indicate that ApoM-HDL or ApoM-S1P may be a potential neuroprotective agent to counteract the cell death induced by brain ischemia.

2. Materials And Methods

2.1. Ethics statement

This study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All animal experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of Changzhou University. The mice were maintained in a room with 12 h light-dark cycle and had constant access to purified water and sterilized food. The neonatal mice were euthanized with carbon dioxide and the cerebral material was obtained 5 min after sacrifice of the animals. Both of male and female neonatal mice were used in this study.

2.2. Cell culture

Cortical astrocytes were isolated and cultured as previously described with modifications (Huang, Li et al. 2014). The neonatal C57BL/6 mice born within 24 h were decapitated. Following removal of the meninges, the cerebral cortices were cut into small pieces and digested with 0.25% trypsin for 20 min at 37 °C. the dissociated cells were incubated in high glucose DMEM (Gibco, Grand Island, USA) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 IU/ml of penicillin and 100 mg/ml streptomycin. The cultures were maintained in a humidified atmosphere (5% CO₂ and 95% air) at 37 °C. After 24 h, the medium was replaced and thereafter twice a week. After 12 days, the confluent cultures were vigorously shaken to remove the microglial cells. The adherent cells were detached and reseeded at a density of 1.5×10^5 cells/ml.

2.3. Oxygen-glucose deprivation

OGD was performed as previously reported with modifications (Huang, Zhang et al. 2008). The astrocytes were rinsed twice and incubated in Earle's solution without glucose. Then the cells were cultured in an anaerobic chamber filled with 95% N₂ and 5% CO₂ at 37 °C. At the end of the treatment, the cultures were returned to normal condition and the medium was changed with a culture medium for indicated time. Prior to OGD treatment, the cells were treated with or for 30 min, and the drugs were continuously applied until the end of recovery. The normoxia controls were washed and incubated with Earle's solution containing 5.6 mM glucose, then cultured under normal condition except OGD treatment.

2.4. siRNA treatment of cells

The astrocytes grown to 70% confluence in 6 well plate before transfection. Thereafter, 100 pmol of S1PR1 or S1PR3 siRNA (Thermo Fisher Scientific Inc., USA) were diluted in 200 ml of Opti-MEM I (ThermoFisher Scientific, USA) and 5 ml of P3000 reagent. Simultaneously, mix 8 ml of Lipofectamine 3000 (ThermoFisher Scientific, USA) and 200 ml of Opti-MEM I solution. Then the duplex siRNA solution was added to the Lipofectamine 3000 solution and incubated for 20 min at room temperature. Thereafter, the siRNA complexes were added to 2 ml of complete medium and the cells were incubated for 16 h at 37 °C in a 5% CO₂ incubator. Then replace the transfection medium with complete medium.

2.5. Purifications of HDL particles with or without ApoM

ApoM-containing HDL was separated by ultracentrifugation followed by immunoaffinity chromatography. Firstly, HDL was isolated from mouse plasma as described with modifications (Christoffersen, Nielsen et al. 2006, Christoffersen, Obinata et al. 2011). A pool of plasma from 10 mice was centrifuged with a Beckman TLA-100 rotor and a Beckman Optima MAX-XP ultracentrifuge (Beckman Coulter, Inc., USA) for 20 h at 50,000 rpm. The density of the solution was adjusted with NaBr and the component with density between 1.063 g/L and 1.21 g/L was collected. The purified HDL was dialyzed against PBS with EDTA at 4°C.

The total HDL obtained from ultracentrifugation was applied to the anti-ApoM column to isolated ApoM-containing HDL. An anti-mouse polyclonal antibody against ApoM (PA5-92403, Thermofisher, USA) was

coupled to 3 ml HiTrap N-hydroxy-succinimide (NHS)-activated columns (Amersham Biosciences) at 0.5 mg/ml gel, according to the manufacturer's instructions. The column was washed with 10 mM Tris-HCl, pH 7.5 with 500 mM NaCl and bound particles were eluted with glycine (0.1 M, pH 2.2). The elution was collected in 1 ml fraction before the flow-through was passed over the column again. The absorbance at 280 nm was determined in each fraction. The HDL preparations were subjected to five rounds of anti-ApoM chromatography until all ApoM particles had been removed.

S1P levels in HDL preparations were quantified by a Sphingosine 1-Phosphate ELISA Kit (Echelon Biosciences Inc., USA). S1P was 0.32 mM/mg of protein in HDL with ApoM. The S1P in HDL without ApoM could not be detected.

2.6. Loading of ApoM with S1P

The ApoM-bound S1P was produced according to the methods previously described (Christoffersen, Obinata et al. 2011). Firstly, S1P was dissolved in methanol followed by evaporation. Then same molar of recombinant murine ApoM (Uscn Life Science Inc., China) was added and sonicated for 3 min in 20 mM Tris-HCl with the pH value of 8.0.

2.7. Cell viability assay

Cell viability was assessed by MTT assay. Briefly, the cells were plated with 1.5×10^5 /ml in 96-well plates. After 24-h incubation, the cells were subject to OGD and recovery. After the treatment, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, USA) was added to each well with the final concentration of 0.5 mg/ml. Following 4-h incubation at 37 °C, the medium was removed and 100 μ l of dimethyl sulfoxide was added to each well. Then the absorbance at 570 nm was measured with a microplate reader (TECAN Infinite F200, Tecan Trading AG, Switzerland). Results were expressed as the percentages of the control.

2.8. Cell death evaluation

The astrocyte death was assessed by the measurement of lactate dehydrogenase (LDH) released into the medium. After the treatments, 50 μ l of supernatants was collected from each well and LDH activity was determined with a LDH assay kit (Roche, USA) according to the manufacturer's instructions.

2.9. Cell apoptosis assay

Cells grown on coverslips were washed with PBS and stained with Hoechst 33258 at 10 mg/mL for 10 min at 37 °C. Thereafter, the cells were observed under a fluorescent microscope (Evos M5000 imaging systems, Thermofisher, USA). The cells with condensed or fragmented nuclei showing strong fluorescence were identified as apoptotic cells. At least 10000 cells were counted in more 3 fields in each coverslip. The cell apoptosis was expressed as percentage of apoptotic cells.

2.10. Immunocytochemistry

Astrocytes seed on coverslips were fixed with 4% paraformaldehyde for 30 min at room temperature and incubated with 5% normal goat serum for 1 h. Thereafter, the cells were exposed to mouse monoclonal anti-GFAP antibody (1: 400, EMD Millipore, USA) at 4 °C overnight. After rinsing with PBS, the cells were incubated with goat anti-mouse Alexa Fluo 488-conjugated secondary antibody (1: 600, Jackson ImmunoResearch Laboratories, USA). The coverslips were mounted with ProLong Gold Antifade Mountant with DAPI (Thermofisher Scientific, USA) and observed under a fluorescent microscope (Evos M5000 imaging systems, Thermofisher, USA).

2.11. Caspase 3 activity assay

The caspase 3 activity was measured with a colorimetric assay kit according to manufacturer's instructions (Abcam). The cell lysates containing 60 mg of protein were incubated with N-acetyl-Asp-Glu-Val-Asp-p-nitroanilide at 200 mM for 2 h at 37 °C. the release of p-nitroanilide was quantified using a microtiter plate reader (Tecan Infinity F50) at 405 nm.

2.12. Protein extraction and electrophoresis

The cells were washed with ice-cold PBS and then lysed in cell lysis buffer (#9803, Cell Signaling Technology, USA) on ice for 30 min. Then, the lysates were centrifuged at 12, 000 ´ g for 30 min at 4 °C and the supernatant were collected as the protein samples. The protein concentration was quantified by a BCA protein assay kit (Pierce, USA) according to manufacturer's instructions. The 10 mg of samples were separated by 10% SDS-polyacrylamide gels. After transferring to nitrocellulose membrane, the blots were blocked with 5% skim milk and incubated with a rabbit monoclonal anti-cleaved Caspase-3 antibody (1: 1000, #9664, Cell Signaling Technology), a rabbit monoclonal anti-Bax antibody (1: 1000, #14796, Cell Signaling Technology), a rabbit monoclonal anti-Bcl-2 antibody (1: 1000, #3498, Cell Signaling Technology), a rabbit monoclonal anti-Erk1/2 antibody (1: 2000, #4695, Cell Signaling Technology), a rabbit monoclonal anti-phospho-Erk1/2 (Thr202/Tyr204) (1:2000, #4370, Cell Signaling Technology), a mouse monoclonal anti-Akt antibody (1:2000, #2920, Cell Signaling Technology), a rabbit monoclonal anti-phospho-Akt (Ser473) antibody (1: 2000, #4060, Cell Signaling Technology), and an mouse monoclonal anti-β-actin antibody (1:3000, B&D Biosciences) at 4 °C overnight. After rinsing several times, the membranes were incubated with a horseradish-conjugated secondary antibody for 1 h at room temperature and exposed on an X-ray film. The results of the protein expression are normalized to β-actin.

2.13. Quantitative real-time PCR (qRT-PCR)

The RNA was isolated with a RNeasy Mini kit including DNase I digestion (Qiagen, USA). The reverse transcription was performed with a High Capacity cDNA Archived Kit (Applied Biosystems, USA) according to the manufacturer's protocol. Then, the real-time PCR analysis was carried out with a detection system (ViiA 7, Applied Biosystems, USA). Amplification of specific PCR products was detected using the SYBR Green PCR Master Mix (Roche, USA). The following primers were used for analysis: mouse S1pr1, 5'-AGCTCAGGGAACCTTTGCGAG-3' and 5'-GAGAAACAGCAGCCTCGCTC-3'; mouse S1pr3, 5'-CTTGACAACGAGAGCCTAT-3' and 5'-GGGAACAATTGGGAGAGTGGT-3'; mouse Cyclophilin, 5'-

GCCAAGACTGAATGGCTGGAT-3' and 5'-CTTGCCATTCCTGGACCCAA-3'. The relative mRNA expression was calculated by using the $2^{-\Delta\Delta CT}$ method with cyclophilin as the reference gene.

2.14. Statistical analysis

Statistical analyses were performed using SPSS 18.0 statistics Software. Data are expressed as means $\pm$ standard deviation ($\bar{x} \pm s$). Difference between groups were examined using a one-way analysis of variance (ANOVA) or student's test (t test). When ANOVA test results for all data were significant, post hoc least significant difference (Bonferonni) was used to determine individual differences. If normality or variance homogeneity were not met, nonparametric tests were applied. A P value <0.05 was considered statistically significant.

3. Results

3.1. Characterization of apoM-containing HDL particles in plasma

To determine the effects of ApoM in HDL on astrocyte cell death, we purified ApoM-containing HDL from plasma. After ultracentrifugation, the isolated HDL was purified on a HiTrap column with a monoclonal antibody against ApoM. Immunoblotting analysis showed that after 5 rounds of purification, ApoM could not be detected in HDL (Fig. 1A), indicating good preparations of ApoM-containing HDL and ApoM-free HDL. In addition, analysis using western blotting against ApoA-I, ApoB and ApoM demonstrated that HDL did not contain ApoB but contain rich ApoA-I (Fig.1B), consistent with the previous findings (Christoffersen, Nielsen et al. 2006). ApoM-containing HDL and ApoM-free HDL contained similar levels of ApoA-I, while HDL contain higher level of ApoA-I, suggesting the partial removal of ApoA-I during the purification processes.

3.2. Astrocytes undergo cell death following ischemic insult *in vitro*

When astrocytes were treated with deprivation of glucose and oxygen, cells underwent cell death (Huang, Zhang et al. 2008). As shown in Fig. 1C, the cell viability was reduced after 4-h OGD treatment, while 2-h OGD did not affect the viability [$F(5, 12) = 85.205$, $P < 0.001$], suggesting the time-dependent effects of OGD on cell death. To assess the cell viability after OGD and recovery, the cells were treated with 4-h OGD and recovery for 24 h, 48 h and 72 h. The results showed that 24-h recovery after 4-h OGD resulted in significant cell death [$F(4, 35) = 66.317$, $P < 0.001$; $F(4, 35) = 118.808$, $P < 0.001$ respectively], as evidenced by MTT reduction assay and LDH assay (Fig. 1D and 1E). As shown in Fig. 1F. after staining with hematoxylin, the astrocytes exposed to 4-h OGD and 24-h recovery exhibited cell injury. Then we choose 4-h OGD/24-h recovery as the treatment for astrocytes in the following study.

3.3. HDL with ApoM protects astrocytes against cell death induced by OGD/recovery.

To assess the role of ApoM-associated HDL in the OGD-induced astrocyte death, mouse HDL⁺ (ApoM-associated HDL) and HDL⁻ (ApoM-depleted HDL) were isolated from C57 mouse plasma. After treated

with OGD in the presence of HDL⁺ or HDL⁻, the cell viability was assessed by MTT assay. As shown in Fig. 2A and 2B, HDL⁺ at 10 ~ 20 mg/ml mitigated cell death and protected against LDH release induced by OGD, while no protective effects were observed for HDL⁻ treatment [$F(4, 35) = 99.296, P < 0.001$; $F(4, 35) = 108.535, P < 0.001$; $F(4, 35) = 183.831, P < 0.001$; $F(4, 35) = 90.188, P < 0.001$ respectively]. In addition, the live cells were counted using trypan blue exclusion test after OGD treatment. As shown in Fig. 2C and 2D, OGD significantly decreased the number of living cells, which was reversed by HDL⁺ at 10 ~ 20 mg/ml [$F(4, 25) = 42.651, P < 0.001$]. Similarly, HDL⁻ at 5 ~ 20 mg/ml did not show any effect [$F(4, 35) = 26.627, P < 0.001$].

3.4. ApoM-associated HDL restores the GFAP expression in astrocytes after OGD and recovery.

Astrocytes are generally more resilient than neurons after ischemic injury due to high level of antioxidant glutathione and less oxidative stress. (Almeida, Delgado-Esteban et al. 2002, Amri, Ghouili et al. 2017) In primary cultured astrocytes, OGD/recovery treatment or oxidative stress also induces significant cell death as well as expression change of GFAP, the specific marker of astrocytes (Chen and Liao 2002, Huang, Zhang et al. 2008, de Pablo, Nilsson et al. 2013), showing the disrupted astrocyte intermediate filament system.

As shown in Fig. 3A, using immunostaining with GFAP antibody, we found that the number of GFAP-positive astrocytes was remarkably decreased after 4 h-OGD and 24 h-recovery. Treatments with HDL⁺ at 10 ~ 20 mg/ml promoted cell survival and increased cell number, while HDL⁻ did not exhibit any protective effects. In addition, immunoblotting analysis revealed that GFAP expression was reduced in the cells after OGD and recovery, which was restored by HDL⁺ but not HDL⁻ (Fig.3B) [$F(6, 35) = 58.014, P < 0.001$].

3.5. ApoM associated HDL protects against astrocyte apoptosis after OGD/recovery.

During cerebral ischemia in mouse brain, astrocytes in the ischemic core undergo apoptosis and dysfunction resulted from oxidative stress, leading to the consequent neuronal death (Liu, Chen et al. 2014, Amri, Ghouili et al. 2017). In order to clarify the role ApoM in the protection by HDL against cell apoptosis, the astrocytes were stained with Hoechst 33258. The apoptotic astrocytes showed condensed nuclei and enhanced blue fluorescence. As shown in Fig. 4A and 4B, the astrocytes exhibited a weak apoptotic ratio but a significantly high apoptotic ration after OGD treatment. HDL⁺ decreased the percentage of apoptotic cells in a dose-dependent manner [$F(6, 35) = 81.542, P < 0.001$; $F(4, 25) = 32.879, P < 0.001$ respectively]. HDL⁺ at 10 ~ 20 mg/ml significantly protected against cell apoptosis compared to OGD only, while 10 ~20 mg/ml of HDL⁻ treatment did not confer protection against cell apoptosis.

Under apoptosis, caspase 3 in cells will be cleaved, leading to the increased expression ratio of Bax and Bcl-2 (Petrache, Fijalkowska et al. 2006). As shown in Fig. 4C and 4D, OGD reduced the expression of Bcl-2 and induced the expression of Bax. Thus, the ratio of Bax/Bcl-2 was enhanced after OGD treatment, and administration of HDL⁺, not HDL⁻, reduced the expression ratio. In addition, the expression of cleaved

caspase 3 (p17) was determined by immunoblotting in the astrocytes. The results showed that caspase 3 was cleaved after OGD treatment, showing the activation of caspase 3. Treatment with 10 mg/ml of HDL⁺ inhibited the cleavage of caspase 3, but HDL⁻ did not show any inhibitory effects [$F(4, 25) = 178.301$, $P < 0.001$; $F(4, 25) = 38.211$, $P < 0.001$ respectively].

3.6. Akt and Erk signaling pathways participate in the anti-apoptotic effects of S1P in astrocytes challenged with OGD

It has been shown that PI3K/Akt and Erk signaling pathways are involved in the anti-apoptotic activities of S1P (Christoffersen, Obinata et al. 2011, Ruiz, Okada et al. 2017). To assess the role of Akt and Erk pathways in our model, we used LY249002 to inhibit PI3K/Akt and PD98059 to inhibit Erk activation. Those inhibitors were added to the astrocytes in the presence of HDL⁺ for 10 min (Akt activation) or 1 h (Erk activation). Then the astrocytes were lysed and the phosphorylation of Akt or Erk protein was analyzed by western blotting. As shown in Fig. 5A and 5B, HDL⁺ induced Akt phosphorylation which was abolished by LY 249002. Similarly, the upregulation of phosphorylated Erk by HDL⁺ was blocked by PD98059 [$F(8, 45) = 124.488$, $P < 0.001$; $F(8, 45) = 101.441$, $P < 0.001$ respectively]. Thereafter, the astrocyte apoptosis after treated with LY249002 or PD98059 in the presence of HDL⁺ was determined by Hoechst 33258 staining. The results showed that blockage of Akt or Erk activation with specific inhibitors abolished the anti-apoptotic effect of HDL⁺ in astrocytes challenged with OGD (Fig. 5C and 5D) [$F(3, 20) = 16.67$, $P < 0.001$]. To confirm the results, we carried out Caspase-3 activity assay and found that HDL⁺ decreased Caspase 3 activity in the cells treated with OGD. However, LY249002 or PD98059 abrogated the protection of HDL⁺ (Fig. 5E) [$F(3, 20) = 28.592$, $P < 0.001$].

It has been reported that ApoM functions as a carrier of S1P in HDL, leading to the activation of downstream signaling of S1P after activating its receptors (Liu, Seo et al. 2014, Blaho, Galvani et al. 2015). In order to directly compare the effects of free S1P and ApoM-bound S1P, we loaded soluble mouse ApoM with S1P to obtain ApoM-bound S1P. Thereafter, we tested the Akt and Erk phosphorylations in the cells after treated with free S1P or ApoM-bound S1P. We found that ApoM-bound S1P induced expression of phosphorylated Akt or ERK comparable to that of free S1P, suggesting the similar effects of S1P bound or unbound to ApoM. However, we did not observe any effects of ApoM on cell apoptosis or Akt activation (data not shown).

3.7. S1pr1 but not S1pr3 is induced in astrocytes after OGD treatment

Upon activation, S1P exerts its biological effects through a group of G-protein coupled receptors, including S1P receptors (S1PR) 1-5. Thus, to determine the participation of S1P receptors in the anti-apoptotic effects of HDL⁺ on astrocytes, the expression of S1prs in astrocytes after OGD were assessed. It has been shown that S1pr1 and S1pr3 are main S1P receptors expressed in astrocytes (Pebay, Toutant et al. 2001), so only these two receptors were analyzed in this study.

After the cells were treated with OGD and recovery, the mRNA levels of *S1pr1* and *S1pr3* were assessed by real-time quantitative RT-PCR. The data showed that the mRNA expression of *S1pr1* in the astrocytes was up-regulated after OGD treatment, while the *S1pr3* level was not changed after OGD (Fig. 6A) [$t(2) = 5.426$, $P < 0.001$; $t(2) = 10$, $P = 0.771$ respectively]. To confirm the results, the protein levels of S1pr1 and S1pr3 were determined by immunoblotting. As shown in Fig. 6B, only S1pr1 protein expression was markedly induced in the astrocytes after OGD treatment [$t(2) = 7.531$, $P < 0.001$; $t(2) = 10$, $P = 0.926$]. These data suggest that S1pr1 may be required for the anti-apoptotic property of HDL⁺.

3.8. Activation of S1pr1 mediates protective effects of ApoM-associated HDL

Next, we determined which S1P subtype receptor is responsible for HDL anti-apoptotic property. Thus, we employed pharmacological antagonists of S1P receptors in the study. In the presence of HDL⁺, a S1pr1 antagonist W146 at 1 mM or a S1pr3 antagonist CAY10444 at 10 mM was added to the culture medium 30 min before OGD and kept in the medium until the end of the experiment. The Hoechst 33258 staining revealed that W146 abolished the protective effect of HDL⁺, while CAY 10444 did not show any effect (Fig. 7A) [$F(2, 15) = 17.764$, $P < 0.001$]. Likewise, as shown in Fig 7B, the reduced Caspase 3 activity after treatment with HDL⁺ was inhibited by S1pr1 inhibition but not by S1pr3 inhibition [$F(3, 20) = 25.754$, $P < 0.001$]. Furthermore, immunoblotting analysis demonstrated that W146 inhibited the upregulation of phosphorylated Akt or Erk1/2, showing the abolishment of Akt/Erk1/2 signaling activation (Fig. 7C) [$F(2, 15) = 121.98$, $P < 0.001$; $F(2, 15) = 114.062$, $P < 0.001$ respectively]. These results suggest that S1pr1 but not S1pr3 activation is required for the anti-apoptotic effect of HDL⁺, which involves the activation of Akt/Erk1/2 signaling pathways.

3.9. Knockdown of S1pr1 abolishes the protection of ApoM-associated HDL

To exclude the possibility of unspecific effects of S1pr inhibitors, specific siRNAs were added to the astrocytes to silence the expression of *S1pr1* and *S1pr3*. After the cells were treated with siRNAs for 3 days, the cell protein extracts were used for analysis of S1pr1 and S1pr3 by immunoblotting. As shown in Fig. 8A, immunoblotting results showed that protein expression of S1pr1 or S1pr3 was reduced by their respective siRNA. Thereafter, the apoptotic astrocytes after treatment with OGD were determined by Hoechst staining. We found that only *S1pr1* silencing mitigated the anti-apoptotic effect of HDL⁺ in astrocytes (Fig. 8B) [$F(3, 20) = 21.869$, $P < 0.001$]. In addition, the Akt and Erk activation were determined by immunoblotting analysis. As shown in Fig. 8C, HDL⁺ or ApoM-bound S1P induced Akt and Erk phosphorylation, while the upregulation of phosphorylated Akt or Erk were blocked after *S1pr1* silencing.

4. Discussion

In the study, we found that ApoM associated HDL exerts protective effects on astrocyte cell death induced by ischemic insult in vitro. The protective effects by HDLs is mediated by the ApoM-S1P complexes. Thus, we connect previous findings and demonstrate that ApoM-containing HDL or S1P in HDLs have anti-apoptotic properties. In addition, we found that S1PR1 activation by S1P and the

downstream Akt/Erk activation participate in the anti-apoptotic effects of HDLs, pharmacological inhibition or genetically knockdown of *S1pr1* abolish the protection of HDLs. Accumulating evidence shows that HDL particles protect cells against cell death induced by endoplasmic reticulum (ER) stress and serum starvation, which is related to its anti-oxidative or anti-inflammatory properties (Blaho, Galvani et al. 2015, Nagao, Toh et al. 2017, Durham, Chathely et al. 2018). Therefore, our results confirmed the protective of HDL particles on astrocytes death after ischemic insults.

S1P is generated through phosphorylation of sphingosine and involved in various processes (Pebay, Toutant et al. 2001, Van Doorn, Van Horssen et al. 2010). Activated platelets, endothelial cells and red blood cells release S1P and contribute to the high levels of S1P in plasma (Gazit, Mariko et al. 2016, Rothhammer, Kenison et al. 2017). Only a small fraction of S1P is available for signaling due to the fact that most of S1P is bound to albumin and HDL (Maceyka and Spiegel 2014, Frej, Mendez et al. 2017). However, reconstituted HDL with exogenous S1P does not show any enhanced anti-apoptotic properties compared with HDL without S1P (de Souza, Vindis et al. 2010), suggesting the S1P bound to ApoM in HDLs accounts for the protection of HDL against cell death. Consistent with these results, we found that HDL containing ApoM protected astrocytes against apoptotic cell death after OGD treatment whereas ApoM-depleted HDL did not show any protection. There are other regulated cell death pathways such as necroptosis, ferroptosis, parthanatos, pyroptosis (Yagami, Yamamoto et al. 2019), contributing to cell death after ischemic insult. Here, we only determined the cell apoptosis that is representative of the pattern of cell death. This is one of the limitations of our work and should be investigated in future work.

In addition, the downregulation of GFAP of the astrocytes was restored by addition of ApoM-bound HDL. It has been reported that S1P bound to albumin also show low activity against endothelial apoptosis (Ruiz, Okada et al. 2017), suggesting the S1P-ApoM complex have the highest affinity to interact with S1PRs. In contrast to the previous report that ApoM-S1P shows higher protective effects (Ruiz, Okada et al. 2017), our data showed that ApoM-S1P complex exhibited similar anti-apoptotic properties compared to free S1P in astrocytes. The results may be explained by the similar activation of S1prs after exposure to ApoM-S1P or free S1P due to the upregulation of *S1pr1*, although free S1P may activate S1PRs less properly than ApoM-S1P complex (Ruiz, Okada et al. 2017). In addition, S1P will be internalized and degraded in cells after binding with S1PRs, which could be mediated by different S1P carrier (Oo, Thangada et al. 2007, Oo, Chang et al. 2011).

The average plasma ApoM concentration is about 0.9 mM and the amount of ApoM in HDL is sufficient to accommodate S1P in HDL particles (Christoffersen, Obinata et al. 2011). Thus, the physiological functions of HDL in blood may be related to the ApoM-bound S1P. S1P binds with its receptors and regulates inflammatory responses via the downstream pathways, such as Akt, NF- κ B and p38 MAPK signaling (Adada, Orr-Gandy et al. 2013, O'Sullivan, O'Sullivan et al. 2018, Liu and Tie 2019). Additionally, S1PR activations participate in the modulation of cell death, such as endothelial cells, resulting from activation of both *S1pr1* and *S1pr3* by ApoM-S1P in HDL (Ruiz, Okada et al. 2017). S1PR1 activation also contributes to the proliferation of astrocytes and pathogenic astrocyte activation (Pebay, Toutant et al. 2001, Rothhammer, Kenison et al. 2017). In this study, we found that *S1pr1*, but not *S1pr3*, was

responsible for the protective effects of HDL particles. S1pr1 expression after OGD was up-regulated while the S1pr3 expression was comparable before and after OGD. Only inhibitor of S1pr1 could abolish the protection by the HDL, while the inhibitor of S1pr3 could not. This suggests that the pattern of S1PR activation to achieve protection may be different depending on tissues.

Akt-Erk signaling plays an important role in mediation of cell injury or cell death. Akt has been found as an oncogene and is activated by many growth factors. Activation of Akt has been proved to inhibit apoptosis induced by serum deprivation, UV irradiation and chemical agents (Kim, Li et al. 2008, Prieto, Cuenca et al. 2010). In endothelial cells, the cytoprotective effects of HDL and S1P come from the activation of Akt pathway, that is, phosphorylation of Akt and Erk (Yang, Lin et al. 2007, Kim, Li et al. 2008). Blockage of Akt signaling with specific inhibitor significantly reduces the protective effects. Activation of Akt also confers protection against ischemic stroke in animals. (Jiang, Li et al. 2015, Zhu, Zhang et al. 2016, Lei and Chen 2018) In our study, the phosphorylation level of Akt was strongly reduced after OGD treatment. ApoM associated HDL or free S1P phosphorylated Akt, while HDL without ApoM did not show any effects on Akt phosphorylation. After Akt phosphorylation, Erk phosphorylation was also observed, indicating the implication of Akt and Erk signaling pathways in the protection of total HDL. Activation of Akt by ApoM associated HDL was strongly suppressed by specific Akt inhibitor which also attenuated the protective effects of ApoM-HDL. Taken together, Akt/Erk signaling may mediate the anti-apoptotic effect of ApoM associated HDL in astrocytes.

5. Conclusions

In summary, HDL containing ApoM induced the activation of Akt/Erk signaling, which conferred protection against the OGD-induced apoptotic death in astrocytes. Thus, pharmacological or genetic activation of Akt or Erk may induce the anti-apoptotic effects after ischemic insults in neuronal cells. These results imply the potential involvement of these pathways by HDL containing ApoM and the complete molecular mechanisms needs to be elucidated. Continued attempts to identify novel target responsible for the Akt activation and to clarify the downstream signaling will pave the way to exploiting therapeutic strategies for the management of cerebral ischemic disorders.

Declarations

CRedit authorship contribution statement

Xiaojia Huang and Linhong Deng performed the research design. Xiaojia Huang, Zhiqi Zhai, Ting Zhou, Chengju Sheng and Chao Zhou conducted the experiments. Xiaojia Huang collected the data and wrote the manuscript. All authors read and approved the manuscript.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Availability of data

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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References

1. Adada M M, Orr-Gandy K A, Snider A J, et al (2013) Sphingosine Kinase 1 Regulates Tumor Necrosis Factor-mediated RANTES Induction through p38 Mitogen-activated Protein Kinase but Independently of Nuclear Factor kappa B Activation. *Journal of Biological Chemistry* 288:27667-27679.
2. Almeida A, Delgado-Esteban M, Bolanos J P, et al (2002) Oxygen and glucose deprivation induces mitochondrial dysfunction and oxidative stress in neurones but not in astrocytes in primary culture. *J Neurochem* 81:207-217.
3. Amri F, Ghouili I, Amri M, et al (2017) Neuroglobin protects astroglial cells from hydrogen peroxide-induced oxidative stress and apoptotic cell death. *J Neurochem* 140:151-169.
4. Blaho V A, Galvani S, Engelbrecht E, et al (2015) HDL-bound sphingosine-1-phosphate restrains lymphopoiesis and neuroinflammation. *Nature* 523:342-346.
5. Cartier A, Leigh T, Liu C H, et al (2020) Endothelial sphingosine 1-phosphate receptors promote vascular normalization and antitumor therapy. *Proceedings of the National Academy of Sciences of the United States of America* 117:3157-3166.
6. Chen C J and Liao S L (2002) Oxidative stress involves in astrocytic alterations induced by manganese. *Exp Neurol* 175:216-225.
7. Christoffersen C, Nielsen L B, Axler O, et al (2006) Isolation and characterization of human apolipoprotein M-containing lipoproteins. *J Lipid Res* 47:1833-1843.
8. Christoffersen C, Obinata H, Kumaraswamy S B, et al (2011) Endothelium-protective sphingosine-1-phosphate provided by HDL-associated apolipoprotein M. *Proc Natl Acad Sci U S A* 108:9613-9618.
9. de Pablo Y, Nilsson M, Pekna M, et al (2013) Intermediate filaments are important for astrocyte response to oxidative stress induced by oxygen-glucose deprivation and reperfusion. *Histochem Cell Biol* 140:81-91.
10. de Souza J A, Vindis C, Negre-Salvayre A, et al (2010) Small, dense HDL 3 particles attenuate apoptosis in endothelial cells: pivotal role of apolipoprotein A-I. *J Cell Mol Med* 14:608-620.
11. Durham K K, Chathely K M and Trigatti B L (2018) High-density lipoprotein protects cardiomyocytes against necrosis induced by oxygen and glucose deprivation through SR-B1, PI3K, and AKT1 and 2.

Biochem J 475:1253-1265.

12. Dusaban S S, Chun J, Rosen H, et al (2017) Sphingosine 1-phosphate receptor 3 and RhoA signaling mediate inflammatory gene expression in astrocytes. *J Neuroinflammation* 14:111.
13. Frej C, Mendez A J, Ruiz M, et al (2017) A Shift in ApoM/S1P Between HDL-Particles in Women With Type 1 Diabetes Mellitus Is Associated With Impaired Anti-Inflammatory Effects of the ApoM/S1P Complex. *Arterioscler Thromb Vasc Biol* 37:1194-1205.
14. Fulmer C G, VonDran M W, Stillman A A, et al (2014) Astrocyte-derived BDNF supports myelin protein synthesis after cuprizone-induced demyelination. *J Neurosci* 34:8186-8196.
15. Galvani S, Sanson M, Blaho V A, et al (2015) HDL-bound sphingosine 1-phosphate acts as a biased agonist for the endothelial cell receptor S1P1 to limit vascular inflammation. *Sci Signal* 8:ra79.
16. Gazit S L, Mariko B, Therond P, et al (2016) Platelet and Erythrocyte Sources of S1P Are Redundant for Vascular Development and Homeostasis, but Both Rendered Essential After Plasma S1P Depletion in Anaphylactic Shock. *Circ Res* 119:e110-126.
17. Huang X, Li Y, Li J, et al (2014) Tanshinone IIA dampens the cell proliferation induced by ischemic insult in rat astrocytes via blocking the activation of HIF-1alpha/SDF-1 signaling. *Life Sci* 112:59-67.
18. Huang X J, Zhang W P, Li C T, et al (2008) Activation of CysLT receptors induces astrocyte proliferation and death after oxygen-glucose deprivation. *Glia* 56:27-37.
19. Jiang Y, Li L, Tan X, et al (2015) miR-210 mediates vagus nerve stimulation-induced antioxidant stress and anti-apoptosis reactions following cerebral ischemia/reperfusion injury in rats. *J Neurochem* 134:173-181.
20. Kappos L, Antel J, Comi G, et al (2006) Oral fingolimod (FTY720) for relapsing multiple sclerosis. *N Engl J Med* 355:1124-1140.
21. Kim J W, Li M H, Jang J H, et al (2008) 15-Deoxy-Delta(12,14)-prostaglandin J(2) rescues PC12 cells from H2O2-induced apoptosis through Nrf2-mediated upregulation of heme oxygenase-1: Potential roles of Akt and ERK1/2. *Biochemical Pharmacology* 76:1577-1589.
22. Kurano M, Hara M, Tsuneyama K, et al (2014) Induction of insulin secretion by apolipoprotein M, a carrier for sphingosine 1-phosphate. *Biochim Biophys Acta* 1841:1217-1226.
23. Lei J and Chen Q (2018) Resveratrol attenuates brain damage in permanent focal cerebral ischemia via activation of PI3K/Akt signaling pathway in rats. *Neurol Res* 40:1014-1020.
24. Liu B, Chen X, Wang Z Q, et al (2014) DNA damage and oxidative injury are associated with hypomyelination in the corpus callosum of newborn Nbn(CNS-del) mice. *J Neurosci Res* 92:254-266.
25. Liu M, Allegood J, Zhu X, et al (2015) Uncleaved ApoM signal peptide is required for formation of large ApoM/sphingosine 1-phosphate (S1P)-enriched HDL particles. *J Biol Chem* 290:7861-7870.
26. Liu M, Seo J, Allegood J, et al (2014) Hepatic apolipoprotein M (apoM) overexpression stimulates formation of larger apoM/sphingosine 1-phosphate-enriched plasma high density lipoprotein. *J Biol Chem* 289:2801-2814.

27. Liu Y and Tie L (2019) Apolipoprotein M and sphingosine-1-phosphate complex alleviates TNF- α -induced endothelial cell injury and inflammation through PI3K/AKT signaling pathway. *Bmc Cardiovascular Disorders* 19:279-287
28. Luscher T F, Landmesser U, von Eckardstein A, et al (2014) High-density lipoprotein: vascular protective effects, dysfunction, and potential as therapeutic target. *Circ Res* 114:171-182.
29. Maceyka M and Spiegel S (2014) Sphingolipid metabolites in inflammatory disease. *Nature* 510:58-67.
30. Mathiesen Janiurek M, Soyulu-Kucharz R, Christoffersen C, et al (2019) Apolipoprotein M-bound sphingosine-1-phosphate regulates blood-brain barrier paracellular permeability and transcytosis. *Elife* 8:pii: e49405.
31. Murthy C R K, Rao K V R, Bai G, et al (2001) Ammonia-induced production of free radicals in primary cultures of rat astrocytes. *Journal of Neuroscience Research* 66:282-288.
32. Nagao M, Toh R, Irino Y, et al (2017) High-density lipoprotein protects cardiomyocytes from oxidative stress via the PI3K/mTOR signaling pathway. *FEBS Open Bio* 7:1402-1409.
33. O'Sullivan S A, O'Sullivan C, Healy L M, et al (2018) Sphingosine 1-phosphate receptors regulate TLR4-induced CXCL5 release from astrocytes and microglia. *Journal of Neurochemistry* 144:736-747.
34. Oo M L, Chang S H, Thangada S, et al (2011) Engagement of S1P(1)-degradative mechanisms leads to vascular leak in mice. *Journal of Clinical Investigation* 121:2290-2300.
35. Oo M L, Thangada S, Wu M T, et al (2007) Immunosuppressive and anti-angiogenic sphingosine 1-phosphate receptor-1 agonists induce ubiquitinylation and proteasomal degradation of the receptor. *Journal of Biological Chemistry* 282:9082-9089.
36. Papadopoulos M C, Koumenis I L, Dugan L L, et al (1997) Vulnerability to glucose deprivation injury correlates with glutathione levels in astrocytes. *Brain Research* 748:151-156.
37. Pebay A, Toutant M, Premont J, et al (2001) Sphingosine-1-phosphate induces proliferation of astrocytes: regulation by intracellular signalling cascades. *Eur J Neurosci* 13:2067-2076.
38. Peng S, Zhao S, Yan F, et al (2015) HDAC2 selectively regulates FOXO3a-mediated gene transcription during oxidative stress-induced neuronal cell death. *J Neurosci* 35:1250-1259.
39. Petrache I, Fijalkowska I, Medler T R, et al (2006) α -1 antitrypsin inhibits caspase-3 activity, preventing lung endothelial cell apoptosis. *Am J Pathol* 169:1155-1166.
40. Prieto P, Cuenca J, Traves P G, et al (2010) Lipoxin A(4) impairment of apoptotic signaling in macrophages: implication of the PI3K/Akt and the ERK/Nrf-2 defense pathways. *Cell Death and Differentiation* 17:1179-1188.
41. Rothhammer V, Kenison J E, Tjon E, et al (2017) Sphingosine 1-phosphate receptor modulation suppresses pathogenic astrocyte activation and chronic progressive CNS inflammation. *Proc Natl Acad Sci U S A* 114:2012-2017.

42. Ruiz M, Okada H and Dahlback B (2017) HDL-associated ApoM is anti-apoptotic by delivering sphingosine 1-phosphate to S1P1 & S1P3 receptors on vascular endothelium. *Lipids Health Dis* 16:36.
43. Tajiri S, Oyadomari S, Yano S, et al (2004) Ischemia-induced neuronal cell death is mediated by the endoplasmic reticulum stress pathway involving CHOP. *Cell Death Differ* 11:403-415.
44. Van Doorn R, Van Horssen J, Verzijl D, et al (2010) Sphingosine 1-phosphate receptor 1 and 3 are upregulated in multiple sclerosis lesions. *Glia* 58:1465-1476.
45. Xu N and Dahlback B (1999) A novel human apolipoprotein (apoM). *J Biol Chem* 274:31286-31290.
46. Yagami T, Yamamoto Y and Koma H (2019) Pathophysiological Roles of Intracellular Proteases in Neuronal Development and Neurological Diseases. *Mol Neurobiol* 56:3090-3112.
47. Yang S L, Lin L, Chen J X, et al (2007) Cytochrome P-450 epoxygenases protect endothelial cells from apoptosis induced by tumor necrosis factor-alpha via MAPK and PI3K/Akt signaling pathways. *American Journal of Physiology-Heart and Circulatory Physiology* 293:H142-H151.
48. Zhu B, Luo G H, Feng Y H, et al (2018) Apolipoprotein M Protects Against Lipopolysaccharide-Induced Acute Lung Injury via Sphingosine-1-Phosphate Signaling. *Inflammation* 41:643-653.
49. Zhu H L, Zhang Y S, Shi Z S, et al (2016) The Neuroprotection of Liraglutide Against Ischaemia-induced Apoptosis through the Activation of the PI3K/AKT and MAPK Pathways. *Scientific Reports* 6:26859.

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