Apolipoprotein M is required for the protective effects of high density lipoprotein against ischemia-induced astrocyte apoptosis

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Running Title: Apolipoprotein M attenuates astrocyte cell death.

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Abstract

Objectives High density lipoprotein (HDL) has been reported to show protective effects against cell death. Apolipoprotein M (ApoM) in HDL can bind with sphingosine-1-phosphate (S1P) and deliver S1P to target cells. This study aimed to evaluate the effects of HDL on astrocyte apoptosis after ischemic insult and determine the role of ApoM.

Methods After ApoM-associated HDL (HDL\textsuperscript{apoM+}) and ApoM-depleted HDL(HDL\textsuperscript{apoM-}) were separated from mouse plasma, primary cultured mouse astrocytes were challenged with oxygen-glucose deprivation followed by recovery in presence of HDL\textsuperscript{apoM+} or HDL\textsuperscript{apoM-}. mRNA and protein samples were collected for biochemical analysis.

Results The addition of HDL\textsuperscript{apoM+} attenuated apoptotic cell death in the astrocytes, but HDL\textsuperscript{apoM-} did not show any effect. S1P receptor 1 (S1PR1) expression was upregulated, and specific S1PR1 inhibitor or genetic knockdown of S1pr1 abolished the protective effects. In addition, activation of Akt and ERK was induced by HDL\textsuperscript{apoM+} or free S1P, and pharmacological inhibition of Akt and ERK reduced the protection of HDL\textsuperscript{apoM+}.

Conclusions ApoM is essential for the protective effects of HDL, which depends on S1PR1 activation and downstream activation of Akt/ERK. Thus, ApoM may be a neuroprotective component in plasma.

Keywords: Apolipoprotein M, sphingosine-1-phosphate, high density lipoprotein, astrocyte, ischemic insult
1. Introduction

Astrocytes are important for the maintenance of cerebral function and homeostasis of blood-brain barrier. Astrocytes can produce free radical scavenger substances and participate in the protection of neurons against oxidative injury [1-3]. Additionally, astrocytes can provide trophic support and physical barrier of glial scar for neurons [4], preventing massive neuronal death. Thus, protection of astrocytes against cell death may be essential for neuronal survival as well as to maintain normal brain function. So far, little is known about the factors in circulation that regulate glial cell survival.

Recently studies have shown that high density lipoproteins (HDL) in blood exhibits therapeutic efficiency for cardiovascular disease [5-7]. Apolipoproteins can bind with different ligands such as oxidized phospholipids and sphingosine 1-phosphate (S1P) [8] and most of S1P is carried by ApoM in blood [9, 10]. Some evidences have showed that ApoM regulates the homeostasis maintenance of endothelial barrier [11, 12]. ApoM knockout mice show decreased HDL-associated S1P concentration in blood and increased vascular permeability [11, 12], implying the regulation of ApoM/S1P axis in the vascular function.

S1P 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 [13, 14]. The most investigated S1PR is S1PR1, which is involved in cell trafficking, angiogenesis and cell survival [7, 11, 14]. 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\textsuperscript{[15]}. Thus, S1PR1 may be a useful therapeutic target for metabolic diseases.

In the study, we investigated the effects of HDL particles on primary cultured mouse astrocytes. We proved that ApoM-containing HDL attenuated astrocyte apoptosis induced by ischemic insult in vitro. The results indicate that ApoM-HDL or ApoM-S1P may be a potential neuroprotective agent to counteract glial death in 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 (approval number: 20-HXJ1 on 1/04/2020). 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\textsuperscript{[4]}. 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 μg/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 × 10⁵ cells/ml.

2.3. Oxygen-glucose deprivation

OGD was performed as previously reported with modifications [16]. 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 μl of Opti-MEM I (ThermoFisher Scientific, USA) and 5 μl of
P3000 reagent. Simultaneously, mix 8 μl of Lipofectamine 3000 (ThermoFisher Scientific, USA) and 200 μl 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 and chromatography. Firstly, HDL was isolated from mouse plasma as described with modifications [17, 18]. 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 and ApoM-depleted 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 HDL solutions were applied to the columns. The elution was collected before the flow-through was passed over the column again. The bound particles were eluted with 0.1 M of glycine with pH 2.2. The
HDL preparations were subjected to several rounds of anti-ApoM chromatography until all ApoM particles had been removed and then used as ApoM-depleted HDL in the study. The elution of bound particles from first two rounds was combined and concentrated by centrifugation for 10 h at 50,000 g at 4°C. Then the solution was used as ApoM-containing HDL.

S1P levels in HDL preparations were quantified by a Sphingosine 1-Phosphate ELISA Kit (Echelon Biosciences Inc., USA). S1P was 0.32 μg/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 [18]. 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 μl 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 μl 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 μg of protein were incubated with N-acetyl-Asp-Glu-Val-Asp-p-nitroanilide at 200 μM 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 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. Ten micrograms of samples were loaded on 10% SDS-polyacrylamide gels. After transferring to nitrocellulose membrane, the blots were blocked with 5% skim milk and incubated with a rabbit monoclonal anti-ApoM antibody (1: 2000, #ab91656, Abcam), a mouse monoclonal anti-ApoAI antibody (1: 200, ab211472, Abcam), a rabbit polyclonal anti-ApoB antibody (1: 200, #ab20737, Abcam), 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), a rabbit monoclonal anti-S1PR1 antibody (1:1000, #ab23386, Abcam), a rabbit monoclonal anti-S1PR3 antibody (1: 5000, #ab108370, Abcam) and an mouse monoclonal anti-β-actin antibody (1:3000, #612656, 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’-AGCTCAGGGAACTTTGCAGG-3’ and 5’-GAGAAACAGCAGCCTCGCTC-3’; mouse s1pr3, 5’-CTTGCAAGACGAGCCATCCTAT-3’ and 5’-GGGAAACATTGGAGGAGGTGTG-3’; mouse cyclophilin, 5’-GCCAAGACTGAATGGCTGGAT-3’ and 5’-CTTGCCATTCTGGACCCCA-3’. The relative mRNA expression was calculated by using the $2^{-\Delta\DeltaCT}$ 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 ± standard deviation (± 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. HDL with ApoM protects astrocytes against cell death induced by OGD/recovery.

To assess the role of ApoM in astrocyte death, mouse HDL<sup>a</sup> apoM<sup>+</sup> and HDL<sup>a</sup> apoM<sup>−</sup> were prepared from mouse plasma. As shown in Figure 1A and 1B, 10 ~ 20 μg/ml of HDL<sup>a</sup> apoM<sup>+</sup> promoted cell survival and protected against LDH release induced by OGD, while no protective effects were observed for HDL<sup>a</sup> apoM<sup>−</sup>. Hematoxylin staining and GFAP immunostaining revealed significant cell death after OGD treatment and the protective effects of HDL<sup>a</sup> apoM<sup>+</sup> (Figure 1C). As shown in 1D, trypan blue exclusion test showed that OGD significantly decreased the number of living cells, which was attenuated by HDL<sup>a</sup> apoM<sup>+</sup> at 10 ~ 20 μg/ml. Similarly, HDL<sup>a</sup> apoM<sup>−</sup> at 5 ~ 20 μg/ml did not show any effect.

3.2. ApoM associated HDL protects against astrocyte apoptosis after OGD/recovery.
During cerebral ischemia, astrocytes in the ischemic core undergo apoptosis and dysfunction resulted from oxidative stress, leading to the consequent neuronal death \cite{19,20}. In order to clarify the apoptotic cell death of astrocytes, the cells were stained with Hoechst 33258. The apoptotic astrocytes showed condensed nuclei and increased nuclei blue fluorescence. As shown in Figure 2A, under normal condition, few astrocytes exhibited condensed nuclei, but a significantly increased cells exhibited blue fluorescent nuclei after OGD treatment. The Figure 2B showed that HDL\textsuperscript{apoM+} reduced the percentage of apoptotic cells in a dose-dependent manner. At 10 ~ 20 \(\mu\text{g/ml}\), HDL\textsuperscript{apoM+} significantly atteinuated cell apoptosis, while HDL\textsuperscript{apoM-} treatment did not show protection.

During cell apoptosis, caspase 3 protein will be cleaved and activated, leading to the increased expression ratio of Bax and Bcl-2 \cite{21}. As shown in Figure 2C and 2D, OGD reduced the expression of Bcl-2 and induced the expression of Bax. Thus, the ratio of Bax/Bcl-2 was increased after OGD treatment, and administration of HDL\textsuperscript{apoM+}, not HDL\textsuperscript{apoM-}, reduced the Bax/Bcl-2 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 \(\mu\text{g/ml}\) of HDL\textsuperscript{apoM+} inhibited the cleavage of caspase 3, but HDL\textsuperscript{apoM-} did not show any inhibitory effects.

4. Discussion
Accumulating evidence shows that HDL particles protect cells against cell death induced by endoplasmic reticulum stress and serum starvation, which is related to its anti-oxidative or anti-inflammatory properties \[22-24\]. However, the component in HDL contributes to its protection remains elucidated. In this study, we purified the HDL\(^{apoM^+}\) and HDL\(^{apoM^-}\) and examined their effects on astrocytes apoptosis induced by ischemic insult in vitro. We found that HDL\(^{apoM^+}\) but not HDL\(^{apoM^-}\) exerted protective effects, suggesting the protective effect of HDL is mediated by the ApoM. S1P or ApoM-bound S1P showed the similar protective effects as HDL\(^{apoM^+}\).

In the current study, we purified the HDL\(^{apoM^+}\) and HDL\(^{apoM^-}\) from mouse HDL and confirmed the removal of ApoM from HDL particles. Interestingly, ApoM can be found in about 5% of HDL particles and a small part of LDL particles (less than 2%), and both HDL and HDL\(^{apoM^-}\) show similar effects on the oxidation \[17\], indicating the anti-oxidative ability of HDL\(^{apoM^-}\). However, we found that HDL\(^{apoM^-}\) did not show any protective effects on astrocyte apoptosis, ruling out the anti-oxidative contribution of HDL. This discrepancy can be explained by that the activity of paraoxonase 1, a major antioxidant enzyme in HDL, is connected with cholesterol efflux of macrophages and protection of lipid oxidation \[25, 26\]. Thus, HDL may target endothelial cells or blood cells and reduce oxidative stress and lipid peroxidation in blood. In addition, the anti-oxidative capacity of HDL may be not sufficient to reverse the oxidative stress induced by OGD treatment in our model.

S1P is generated through phosphorylation of sphingosine \[13, 27\] and only a small fraction of S1P is available for signaling because that most of S1P is bound to albumin
and HDL [28, 29]. The gene dosage of ApoM dominates the S1P content in plasma, evidenced by that the ApoM knockout mice exhibit two-fold decrease of S1P content, while ApoM transgenic mice show increased plasma S1P [18]. The amount of ApoM in HDL is sufficient to accommodate S1P in HDL particles and the lipoprotein-bound S1P can be only found in apoM-associated HDL in human plasma [18]. However, reconstituted HDL with exogenous S1P does not show any enhanced anti-apoptotic properties compared with HDL without S1P [30], suggesting the S1P bound to ApoM in HDL particles accounts for the protection of HDL against cell death. Thus, increase of ApoM-bound S1P in HDL may be a potential therapeutic strategy for ischemic neuronal death.

Upon activation, S1PRs regulate inflammatory responses via the downstream pathways, including Akt, nuclear factor-κB and p38 mitogen-activated protein kinase signaling [31-33]. Additionally, S1PRs participate in the modulation of cell death, resulting from activation of both S1PR1 and S1PR3 by ApoM-S1P in HDL [7]. S1PR1 activation also contributes to the proliferation of astrocytes and pathogenic astrocyte activation [27, 34]. In this study, we found that S1PR1, but not S1PR3, was responsible for the protective effects of HDLapoM+ particles. Our data ties ApoM-mediated protection against astrocyte apoptosis with S1PR1 only. S1PR1 regulates vascular permeability, which may be linked with Rho superfamily of small GTPases, because of the preferential activation of Rac and Rho upon S1PR1 activation [35, 36]. Rho family modulate cytoskeleton dynamics and assembly [37, 38]. On the contrary, inhibition of Rac downstream of S1PR1 results in actin depolymerization and disruption of vascular
junction\textsuperscript{[37]}. These results suggest the different functions and downstream pathway
between S1PR1 and the other S1PRs.

Several lines of epidemiological evidences show the relationship of low serum concentrations of HDL with higher risk of stroke, likely due to the atheroprotective and anti-inflammatory properties of HDL\textsuperscript{[39]}. Intracerebral infusion of human plasma HDL attenuates neuronal injury and contributes to myelination and oligodendrogenesis in the ischemic brain after stroke\textsuperscript{[40, 41]}. In together with those results, this study raises a possible hypothesis about the protection of ApoM in cerebrospinal fluid in the stroke.

Considering the complication of pathological courses of astrocyte injury following ischemia, there are some limitations in the current study. The first one is that we only determined the cell apoptosis, which is one of the cell death pathways. The other pathways include necroptosis, necrosis, pyroptosis and so on. More studies are required to explore the possible cell death pathways involved. Secondly, in vivo model needs to be set up to verify the protection of HDL\textsuperscript{apoM+}, indicating the future direction for our future studies.

5. Conclusions

In summary, ApoM is required for HDL-induced activation of Akt/ERK signaling, which protects against the ischemia-induced apoptotic death in astrocytes. Thus, modulation of ApoM-HDL particles in blood may promote beneficial effects on neuronal cells after brain ischemia. These results imply the potential involvement of these pathways by HDL-containing ApoM in the therapy of stroke. 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.

**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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**Figure legends**

**Figure 1** Treatment with HDL\(^{\text{ApoM}^+}\) protects against astrocyte death after 4-h OGD and 24-h recovery. (A) MTT reduction assay revealed that HDL\(^{\text{apoM}^+}\) increased cell viability while HDL\(^{\text{ApoM}^-}\) did not show any effects. (B) LDH release assay showed that HDL\(^{\text{apoM}^+}\) protected against cell death. (C) Hematoxylin staining images and GFAP immunostaining images of the astrocytes showed HDL\(^{\text{apoM}^+}\) not HDL\(^{\text{ApoM}^-}\) enhanced cell survival after OGD and recovery. (D) Trypan blue exclusive assay showed that only HDL\(^{\text{apoM}^+}\) mitigated cell death. Data are expressed as mean ± SD; n = 6; *P < 0.05, **P < 0.01 compared with OGD treatment.

**Figure 2** HDL\(^{\text{apoM}^+}\) inhibits astrocyte apoptosis induced by OGD treatment. (A) Representative Hoechst 33342 staining images showed that HDL\(^{\text{apoM}^+}\) at 10 µg/ml inhibited cell apoptosis in the astrocytes. (B) Quantification of apoptotic cells determined by Hoechst 33342 staining analysis. (C) Western blotting analysis revealed the decreased Bax/Bcl-2 expression ratio and cleaved caspase 3 expression in the astrocytes after HDL\(^{\text{apoM}^+}\) treatment. Data are expressed as mean ± SD; n = 4; **P < 0.01 compared with OGD treatment.
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