Acetylpuerarin inhibits oxygen-glucose deprivation-induced neuroinflammation of rat primary astrocytes via the suppression of HIF-1 signaling

YANXIAO XIANG1, PENGCHAO DU2, XIUMEI ZHANG3, SIDHARTHA BISWAS4, GUANGJUN JIAO5 and HAICHUN LIU5

1Department of Clinical Pharmacy, Qilu Hospital of Shandong University, Jinan, Shandong 250012; 2College of Basic Medicine, Binzhou Medical University, Yantai, Shandong 264033; 3Department of Pharmacology, Shandong University School of Medicine; Departments of 4Hepatobiliary Surgery and 5Orthopaedics, Qilu Hospital of Shandong University, Jinan, Shandong 250012, P.R. China

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Abstract. In the central nervous system (CNS), ischemic injury induced by inflammation associated with astrocytes serves an important role in physiological and pathological processes. Neuroinflammation leads to the release of pro-inflammatory cytokines, including tumor necrosis factor-α and interleukin-1β. The aim of the present study was to investigate whether acetylpuerarin attenuates oxygen-glucose deprivation (OGD)-induced astrocyte inflammation and secretion of pro-inflammatory cytokines via inhibiting hypoxia-inducible factor-1 (HIF-1) activation and suppressing downstream primary astrocyte signaling in rats. The results demonstrated that acetylpuerarin attenuates astrocyte viability and induces morphological changes following OGD stress. Furthermore, acetylpuerarin suppresses the stimulation of HIF-1α and nuclear factor (NF)-κB signaling pathways, while attenuating the expression and secretion of pro-inflammatory cytokines via HIF-1 suppression in OGD-induced astrocytes. These findings indicate that acetylpuerarin may attenuate OGD-induced astrocyte damage and inflammation in rat primary astrocytes via suppressing HIF-1 activation and NF-κB signaling. These results suggest that acetylpuerarin regulates inflammation associated with astrocytes and may represent a novel therapeutic agent for the treatment of neuroinflammation in the CNS.

Introduction

Astrocytes are important cells in the central nervous system (CNS) that serve a key role in the inflammatory response. Astrocytes exhibit complex functions in ischemic injury associated with the CNS due to the secretion of large volumes of anti-inflammatory and pro-inflammatory cytokines in response to changes in the cellular microenvironment (1). In the early stages of ischemic injury associated with the CNS, astrocytes may be activated to secrete anti-inflammatory and neuroprotective cytokines. Furthermore, as ischemic injury progresses and neuroinflammation increases, astrocytes may be damaged and subsequently release pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-6 (2).

In the present study, hypoxia-ischemia conditions were induced via oxygen-glucose deprivation (OGD) in vitro. Hypoxia-inducible factor-1 (HIF-1), an important regulator of oxygen homeostasis, is a heterodimeric transcription factor comprising HIF-1α and HIF-1β. HIF-1α may only be detected at low levels due to its continuous degradation; however, HIF-1β remains stable constitutively (3). Formation of the active HIF-1 complex is induced by the stabilization and accumulation of HIF-1α protein under hypoxic conditions. Following HIF-1 activation, the transcription of hypoxia-inducible genes associated with angiogenesis, vasodilation and cell survival is increased (2). HIF-1 also serves an important role in inflammatory and immune responses. Immunomodulatory cytokines, including TNF-α and IL-1, upregulate HIF-1-dependent gene expression (4). HIF-1 activation is associated with the phosphoinositide-3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) signaling pathways. Furthermore, HIF-1 subunits interact with heat shock proteins and other co-factors (4). HIF-1 upregulates a number of proteins that increase inflammation and blood flow, including nitric oxide synthase (NOS), cyclooxygenase-2 (COX-2), vascular endothelial growth factor and heme oxygenase-1 (4). In addition, HIF-1 activation serves a crucial role in inflammatory responses (4). However, the
exact role of HIF-1 in neuroinflammation has not yet been determined.

Acetylpuerarin is a novel modified isoflavone derivative of puerarin. Puerarin is an important active isoflavone glycoside extracted from the roots of *Pueraria lobata* and has been widely used in China for the treatment of ischemic strokes (5). The effect of acetylpuerarin on signaling pathways associated with HIF-1-regulated inflammation in astrocytes remains unclear. The present study therefore aimed to investigate the effect of acetylpuerarin and HIF-1 regulation on OGD-induced inflammation in astrocytes.

**Materials and methods**

**Primary astrocyte extraction and culture.** Primary astrocytes were isolated from 1-day-old neonatal Wistar rats as previously described (6). All experimental animals were obtained from the Laboratory Animal Center of Shandong University (Jinan, China). Cortical tissues were mechanically dissociated in PBS, and astrocytes were seeded at 1x10⁶ cells/ml in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.). Cells were incubated with serum-free DMEM at 37°C for 1 h prior to further experimentation. Primary astrocytes were divided into three groups: Acetylpuerarin (AP)+OGD, OGD and control groups. The current study was approved by the Scientific Research Ethics Committee of Qilu Hospital of Shandong University (Jinan, China).

**OGD-induced inflammation of astrocytes.** Astrocytes were seeded in 6-well plates at a density of 5x10⁴ cells/well with 1 ml DMEM containing 10% FBS. The medium was subsequently replaced with fresh serum-free medium and plates were incubated at 37°C for 24 h. Astrocytes were pre-treated with or without acetylpuerarin (1.6 µM) for 24 h, as previously described (7). Cells in the AP+OGD and OGD groups were incubated with OGD stimulation at 37°C for 2 h in a hypoxic incubator containing 94% N₂, 1% O₂ and 5% CO₂ (8) with the following isotonic OGD solution (1 ml; pH 7.4): 0 mM glucose, 21 mM NaHCO₃, 120 mM NaCl, 5.36 mM KCl, 0.33 mM Na₂HPO₄, 0.44 mM KH₂PO₄, 1.27 mM CaCl₂, and 0.81 mM MgSO₄ (9). Normoxic control cells were incubated in 5% CO₂ and atmospheric air in an isotonic control solution for 2 h at 37°C.

**Immunocytochemistry of astrocytes.** Astrocytes were collected and subcultured on sterile glass coverslips at 37°C for 12-16 h. Following fixation with 4% formaldehyde for 15 min at room temperature and permeabilization using Triton-X-100 for 20 min, all astrocytes were incubated with primary antibodies (rabbit anti-GFAP antibody; cat. no. ab7260; 1:1,000; Abcam, Cambridge, MA, USA) at 4°C for 12 h. PBS was used to wash the cells, which were subsequently incubated with secondary antibodies (goat anti-rabbit secondary antibodies; cat. no. A-21094; Alexa Fluor 633; 1:30,000; Invitrogen; Thermo Fisher Scientific, Inc.) for 40 min at 37°C. Images were captured at a magnification of x200 using a Nikon Eclipse 80i fluorescence microscope (Nikon Corportaion, Tokyo, Japan) and analyzed using ImageJ 2X software (National Institutes of Health, Bethesda, MD, USA).

**Western blot analysis.** The immunoreactivity of HIF-1α and nuclear factor (NF)-κB p65 was investigated using western blotting post-OGD stimulation for 2 h. Cells were washed using PBS and collected in a radioimmunoprecipitation lysis buffer (Betotime Institute of Biotechnology, Haimen, China) containing 1 mM PMSF. Nuclear extraction was performed as previously described (10). Protein extraction was performed using a BCA kit (Betotime Institute of Biotechnology) and adjusted to the same concentration. Protein (20 µg) was loaded per lane and polyvinylidene membranes were used for protein transfer. Primary antibodies (anti-HIF-1α; cat. no. 36169; 1:1,000) and anti-NF-κB (cat. no. 8242; 1:1,000; each, Cell Signaling Technology, Inc., Danvers, MA, USA) and secondary antibodies (mouse anti-rabbit IgG mAb #5127, 1:30,000 dilution, Cell Signaling Technology, Inc., Danvers, MA, USA) were used. β-actin and histone H1 (anti-β-actin, cat. no. ab8226; 1:1,000; anti-histone H1, cat. no. ab203337; 1:1,000; Abcam) were used as loading control. 5% BSA was used for blocking at room temperature for 1 h. Immunoblots were visualized using a FluorChem E Chemiluminescent Western Blot Imaging system (ProteinSimple, San Jose, CA, USA) and ImageJ 2X (National Institutes of Health) was used to quantify the results. The relative expression of target proteins was determined relative to a control (β-actin or histone H1).

**ELISA.** Following OGD stimulation for 2 h, the immunoreactivity and expression of IL-1β and TNF-α in the astrocyte culture medium were determined using ELISA kits (Rat IL-1β/IL-1F2 Quantikine ELISA kit, cat no. RB00C; Rat TNF-α Quantikine ELISA kit, cat. no. RTA00; each, R&D Systems Inc., Minneapolis, MN, USA). A Varioskan Flash Multimode Reader was used to detect optical density values at 450 nm.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Astrocytes were pre-treated with or without acetylpuerarin for 24 h, followed by OGD induction for 2 h. Total RNA was extracted by using TRIzol Plus.
RNA Purification kit (cat. no. 12183555; Invitrogen, Thermo Fisher Scientific, Inc.). 2^ΔΔCq was used to quantify the levels of mRNA expression following a protocol as previously described (7). PCR conditions and primers used for amplification were performed according to the manufacturer’s protocol using a SYBR GreenER qPCR SuperMix (cat. no. 11761500; Invitrogen; Thermo Fisher Scientific, Inc.) as previously described (7). Lightcycler Software version 4.0 (Roche Applied Science) was used for quantitative data analysis. The relative expression of target genes was normalized to β-actin expression. Experiments were performed in triplicate.

Statistical analysis. All quantitative data are presented as the mean ± standard deviation. Statistical analysis was performed using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). A Student’s t-test was performed for pairwise comparisons. Variance between multiple groups was analyzed using one-way analysis of variance followed by a Dunnett’s test for comparisons. P<0.05 was considered to indicate a statistically significant difference.

Results

Acetylpuerarin rescues astrocyte morphology under OGD stress. Following OGD induction for 24 h, the outline and appearance of astrocytes were observed to be thinner, and the surface area of astrocytes (µm²/cell) was significantly decreased (369.52±16.67) compared with the control group (442.28±18.68; Fig. 1A-D; P<0.05). In addition, the integrated optical density (IOD) of glial fibrillary acidic protein (GFAP) was revealed to be significantly decreased (612.1±86.7) compared with the control group (732.3±98.27; Fig. 1E; P<0.05).

Figure 1. Astrocyte morphology under OGD stress detected by immunocytochemistry. (A) AP+OGD group, (B) OGD group, (C) control group. The outline and appearance of astrocytes is thinner in the OGD group. (D) Area of astrocytes (µm²/cell) in each group. (E) IOD of astrocytes in each group. *P<0.05. AP, acetylpuerarin; OGD, oxygen-glucose deprivation; GFAP, glial fibrillary acidic protein; IOD, integrated optical density.
decreased in the OGD group (438.60±16.37) compared with the control group (531.35±38.41; P<0.05; Fig. 1E). However, the morphology of astrocytes was markedly improved in the AP+OGD group compared with the OGD group (Fig. 1A-C). Furthermore, the area of astrocytes significantly increased to 437.09±23.66 (Fig. 1D) and the IOD of GFAP increased significantly to 520.26±21.33 following treatment with acetylpuerarin compared with the OGD group (P<0.05; Fig. 1E).

Acetylpuerarin attenuates OGD-induced astrocyte damage. The effect of acetylpuerarin on OGD-induced astrocytes was investigated using MTT and LDH release assays. The results of the MTT assay demonstrated that astrocyte viability was significantly decreased following OGD induction (0.25±0.04) compared with the control group (0.65±0.08; P<0.01); however, pre-treatment with acetylpuerarin was revealed to significantly attenuate this effect (0.46±0.06; P<0.05; Fig. 2A).

LDH release assays were performed to investigate cell damage in the different groups. The percentage of LDH released by the control group was 2.39±0.81%; whereas in the OGD group this value was 31.66±3.71% (P<0.01 vs. control group), suggesting that OGD induced cell damage in astrocytes. However, treatment with acetylpuerarin significantly reduced the amount of LDH released in the AP+OGD group (18.67±4.8; P<0.05 vs. OGD group), suggesting that acetylpuerarin suppresses OGD-induced cell damage in astrocytes (Fig. 2B).

Acetylpuerarin decreases HIF-1α and NF-κB p65 expression in OGD-induced astrocytes. To investigate the effect of acetylpuerarin on OGD-induced neuroinflammation, HIF-1α and NF-κB p65 expression in OGD-induced astrocytes was determined following pre-treatment with acetylpuerarin using western blot analysis. The results demonstrated that
HIF-1α was significantly upregulated in the OGD group (0.91±0.13) compared with the control group (0.29±0.05; \( P<0.01 \); Fig. 3A and B). However, when cells were pre-treated with acetylpuerarin, HIF-1α was significantly downregulated in the AP+OGD group (0.57±0.06) compared with the OGD group (\( P<0.05 \); Fig. 3A and B). Furthermore, NF-κB p65 was upregulated in OGD-induced astrocytes (0.89±0.10) compared with the control (0.32±0.04; \( P<0.01 \)), and this effect was significantly attenuated in the AP+OGD group (0.57±0.08) compared with the OGD group (\( P<0.05 \); Fig. 3A and C). These results suggest that acetylpuerarin decreases the activation of NF-κB, an important regulator of pro-inflammatory pathways, via suppressing HIF-1α activation in OGD-induced astrocytes.

Acetylpuerarin attenuates inflammatory cytokine secretion in astrocytes induced by OGD. TNF-α and IL-1β expression in the astrocyte culture medium was measured using ELISA following 2 h of OGD induction. The results revealed that OGD treatment significantly increased the expression of TNF-α in astrocytes (1,582.96±169.14 pg/ml) compared with the control group (52.88±20.81 pg/ml; \( P<0.01 \)), and that this was significantly attenuated in acetylpuerarin pre-treated astrocytes (1,058.76±154.97 pg/ml; \( P<0.05 \) vs. OGD group; Fig. 4A). IL-1β was also significantly upregulated following OGD induction in astrocytes (324.47±46.69 pg/ml) compared with the control group (28.97±14.82 pg/ml; \( P<0.01 \)), and this effect was significantly attenuated in astrocytes pre-treated with acetylpuerarin (134.06±23.66 pg/ml; \( P<0.01 \) vs. OGD group; Fig. 4B).

**Acetylpuerarin suppresses the mRNA expression of pro-inflammatory cytokines in astrocytes induced by OGD.** It is well established that astrocytes serve a role in normal and abnormal processes associated with the CNS via the release of cytokines (1,11). Therefore, the expression of pro-inflammatory cytokines following OGD induction was measured using RT-qPCR. Acetylpuerarin pre-treatment significantly attenuated the OGD-induced upregulation of TNF-α (7.18±0.59; \( P<0.01 \)) and IL-1β (13.21±2.15; \( P<0.01 \)) mRNA expression (Fig. 4C and D).

**Discussion**

Astrocytes are multifunctional glial cells that serve important roles in neurogenesis and neuron repair in the CNS. During ischemic injury of the brain, astrocytes may protect and repair damaged neurons (12,13). Furthermore, the microenvironment of astrocytes may be modified by numerous pro-inflammatory and anti-inflammatory cytokines secreted by astrocytes (14). Glial cells regulate inflammatory processes associated with the production of immunomodulatory molecules, phagocytosis of cellular debris and recruitment of immune cells from the peripheral blood (1). Although glial cell activation is essential for the maintenance of neuronal function under stress or pain conditions, an excessive response induces cell damage and hinders regeneration in the injured CNS (15). HIF-1 is...
a dimeric transcriptional complex that serves a key role in maintaining oxygen and energy homoeostasis, and in immune responses (16). Immunomodulatory cytokines, including IL-1 and TNF-α, are able to stimulate HIF-1-dependent gene expression, even in normoxic cells (17). In addition, the activation of HIF-1 has been reported to be associated with the PI3K and MAPK signaling pathways (17). HIF-1 increases the transcription of several proteins associated with inflammation; for example, endothelial and inducible NOS and COX-2 upregulate pro-inflammatory cytokine expression, which propagates neuroinflammation.

Our previous study demonstrated that acetylpuerarin inhibits lipopolysaccharide (LPS)-induced arachidonic acid (AA)-metabolizing enzymes and AA metabolites in astrocytes via downregulating secretory phospholipase A2 (sPLA2), as well as phosphorylating extracellular signal-regulated kinase (ERK)1/2, cytosolic phospholipase A2α (cPLA2α) and NF-κB. Furthermore, our previous study revealed that acetylpuerarin inhibits the LPS-induced activation of NF-κB and ERK1/2, as well as the expression of important regulatory enzymes in primary rat astrocytes, including sPLA2, cPLA2α, COX-2 and 5-lipoxigenase. These reports indicate that acetylpuerarin possesses anti-inflammatory properties; however, the underlying mechanism remains unclear. As such, the effect of acetylpuerarin on HIF-1α and associated signaling pathways was investigated in the present study. The results revealed that OGD significantly induces astrocyte damage and morphological changes, while treatment with acetylpuerarin attenuates these changes. Furthermore, the results demonstrated that acetylpuerarin suppresses OGD-induced HIF-1α and NF-κB expression in astrocytes. In addition, HIF-1 is associated with MAPK signaling and increased NOS and COX-2 expression, which in turn stimulates the downstream inflammatory response.

In conclusion, the results of the present study indicate that acetylpuerarin attenuates the inflammatory response and the secretion of pro-inflammatory cytokines in OGD-induced astrocytes. Furthermore, it was demonstrated that acetylpuerarin suppresses HIF-1α expression and NF-κB activation. However, whether acetylpuerarin protects astrocytes from ischemic injury and neuroinflammation via the HIF-1α and NF-κB signaling pathways in vivo remains unclear. Future studies should investigate the underlying regulatory mechanism of acetylpuerarin associated with neuroinflammation in astrocytes in vivo, utilizing animal models to investigate neurological diseases.

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Availability of data and materials

The analyzed data sets generated during the present study are available from the corresponding author on reasonable request.

Authors’ contributions

YX designed the present study, performed cell culture and wrote the manuscript; PD performed cell intervention and immunocytochemistry; XZ performed MMT and LDH assays and acted as the advisor for other experiments; SB performed statistical analysis of data and wrote the manuscript; GJ performed western blotting, RT-qPCR and ELISA; and HL supervised the present study and performed statistical analysis.

Ethics approval and consent to participate

The current study was approved by the Scientific Research Ethics Committee of Qilu Hospital of Shandong University (Jinan, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References