Resveratrol attenuates hypoxia-induced neurotoxicity through inhibiting microglial activation

Qun Zhang a,1, Lin Yuan a,1, Qingrui Zhang a, Yan Gao a, Guangheng Liu a, Meng Xiu b, Xiang Wei a, Zhen Wang a,**, Dexiang Liu b,**

a Department of Physiology, Shandong University School of Medicine, Jinan, Shandong 250012, PR China
b Department of Medical Psychology, Shandong University School of Medicine, 44#, Wenhua Xi Road, Jinan, Shandong 250012, PR China

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Resveratrol is a natural polyphenol enriched in Polygonum cuspidatum and has been found to afford neuroprotective effects against neuroinflammation in the brain. Activated microglia can secrete various pro-inflammatory cytokines and neurotoxic mediators, which may contribute to hypoxic brain injuries. The aim of this study is to investigate the potential role of resveratrol in attenuating hypoxia-induced neurotoxicity via its anti-inflammatory actions through in vitro models of the BV-2 microglial cell line and primary microglia. We found that resveratrol significantly inhibited hypoxia-induced microglial activation and reduced subsequent release of pro-inflammatory factors. In addition, resveratrol inhibited the hypoxia-induced degradation of IκB-alpha and phosphorylation of p65 NF-κB protein. Hypoxia-induced ERK1/2 and JNK phosphorylation was also strongly inhibited by resveratrol, whereas resveratrol had no effect on hypoxia-stimulated p38 MAPK phosphorylation. Importantly, treating primary cortical neurons with conditioned medium (CM) from hypoxia-stimulated microglia induced neuronal apoptosis, which was reversed by CM co-treated with resveratrol. Taken together, resveratrol exerted neuroprotection against hypoxia-induced neurotoxicity through its anti-inflammatory effects in microglia. These effects were mediated, at least in part, by suppressing the activation of NF-κB, ERK and JNK MAPK signaling pathways.

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1. Introduction

Microglial cells are the resident macrophage-like population within the central nervous system (CNS) and they are now recognized as the prime component of the brain immune system. In normal conditions, microglia actively survey the microenvironment and ensure normal CNS activity by secreting neurotrophic factors such as neuronal growth factor, and reactive oxygen species. Although microglial activation plays an important role in phagocytosis of dead cells in the CNS, over-activated microglia cause inflammatory responses leading to neuronal and axonal degeneration, oligodendrocyte death as well as disruption of the immature blood brain barrier. Recent evidence has indicated that inflammatory mediators like interleukin (IL)-1beta, tumor necrosis factor-alpha (TNF-alpha), nitric oxide (NO), monocyte chemoattractant protein-1 and macrophage colony stimulating factor which are produced by activated microglia are linked to the pathogenesis of periventricular white matter damage in the hypoxic brain [1]. Thus, pharmacological interference with the overactivation of microglia may have a therapeutic benefit in the treatment of hypoxic damage.

Resveratrol (trans-3,4,5-trihydroxystilbene) is a phenolic compound enriched in Polygonum cuspidatum and also found abundantly in the skin of red wine and red grapes [2]. Numerous studies have demonstrated that resveratrol exerts a variety of bioactivities, such as anti-inflammatory, anticancer, antioxidant and antiapoptotic [3–5]. Importantly, resveratrol can traverse through the blood brain barrier to act as a powerful neuroprotective agent in vitro in cell lines and in vivo in animals. It was demonstrated that resveratrol protected cortical neurons from oxidative stress-induced injury [6] and inhibited the β-amyloid (Aβ1-42) or ethanol-induced toxicity in the PC12 cells [7,8]. Resveratrol suppressed alcohol-induced cognitive deficits and neuronal apoptosis [9]. In addition, resveratrol has
been found to reduce the production of IL-1 beta and TNF-alpha induced by LPS or Aβ in the microglia [10,11]. Further studies showed that the powerful neuroprotective effect of resveratrol has also been confirmed in neurodegenerative disorders, such as Parkinson’s disease, Alzheimer’s disease [12,13] and in traumatic brain injury [14] or cerebral ischemia [15].

Considering that inflammatory response is involved in the pathophysiology of hypoxia brain injuries, and there exist no studies examining the effects of resveratrol on these targets, it is important to delineate the precise neuroprotective mechanism of resveratrol. To this end, we tested the effects of resveratrol on hypoxia-induced neurotoxicity and microglial activation using the BV-2 microglial cell line. To assess the underlying molecular mechanisms of the anti-inflammatory properties of resveratrol, we evaluated its effects on the activity of nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK).

2. Materials and methods

2.1. Cell culture and hypoxic exposure

BV-2 cells in a 5% CO2 incubator were maintained in Dulbecco’s modified Eagle medium (DMEM, Hyclone Co., Logan, UT, USA) with 10% fetal bovine serum (FBS, Hyclone Co.), 2 mM l-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich, St Louis, MO, USA). For all experiments, BV-2 cells were used at 75–80% confluency. Prior to use in the experiment, plated cells were incubated with serum-free DMEM for 1 h, and then the medium was replaced with serum-free DMEM containing resveratrol (Sigma-Aldrich) for the various time intervals and concentrations as indicated below. Resveratrol was initially dissolved in normal saline. For most experiments, BV-2 cells were per-treated with resveratrol for 30 min followed by hypoxia for 24 h, while controls were treated with the vehicle (normal saline) except where indicated differently. The NF-κB inhibitor-PDTC was obtained from Sigma-Aldrich. The ERK inhibitor-PD98059 and JNK inhibitor-SP600125 were obtained from Beyotime (Shanghai, China). Lenalidomide was obtained from AbMole BioScience (Houston, TX, USA).

Primary microglia were prepared as described previously [16]. Briefly, from the cerebral cortices of mice, aged 1–2 days, devoid of meninges and blood vessels, were dissociated by mild mechanical trituration. The isolated cells were cultured for 14 days in DMEM/F12 (HyClone Co.) supplemented with 10% FBS (HyClone Co.). Then the mixed glial cultures were shaken on an orbital shaker at 250 rpm for 2 h to dislodge microglial cells. Cells were cultured for 7 days before treatment. The experimental protocol was approved by the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985), and efforts were engaged to minimize the number of animal usage and suffering.

Cells were challenged to hypoxia by placing them in a chamber (Model: Heraeus HERAcell 240i; Thermo Scientific, USA) (3% oxygen + 5% CO2 + 92% nitrogen) at 37 °C for the time intervals indicated below. Cells serving as controls were incubated at 37 °C with 95% air and 5% CO2 [17].

2.2. Cell viability assay

BV2 cells were seeded in 96-well culture plates at a density of 5 × 10^4 cells/well. Cell proliferation was analyzed at 24 and 48 h after

![Fig. 1. Effects of resveratrol treatment on cell viability in microglia. (A) BV-2 cells maintained in serum-free medium exposure to hypoxia were incubated in the absence or presence of indicated concentrations of resveratrol (Res) (0–1000 nM) for 24 h and 48 h, and cell viability was examined by MTT assay. Values of cell viability were expressed as a percentage relative to those obtained in controls. (B) Morphological changes of BV2 cells and primary microglia exposed to hypoxia with or without resveratrol (10 nM) co-treatment. Scale bar = 50 μm. Images are representative of triplicate sets. Note the change in external morphology of microglia bearing long extending and stout processes after hypoxia insult. Values represent the mean ± SD of three independent experiments. “p < 0.01, “p < 0.001 Hypoxia (Hy) vs Control (Con); #p < 0.05, ##p < 0.01, ###p < 0.001 Hy + Res vs Hy.”]
hypoxia with or without differing concentration of resveratrol, using the MTT assay. A volume of 20 μl MTT solution (5 mg/ml) was added to each well, and the cells were incubated for another 4 h in a humidified incubator at 37 °C. Then, after removing supernatant, 200 μl of dimethylsulfoxide was added to each well and mixed thoroughly for 10 min. The optical density (OD) was measured at 490 nm. Cell viability was expressed as a percentage of viable cells obtained relative to that of controls.

2.3. Measurement of IL-1 beta and TNF-alpha

Detection of IL-1beta and TNF-alpha in the supernatant of treated and untreated BV2 cells cultures was determined with a mouse IL-1beta and TNF-alpha enzyme-linked immunosorbent assay (ELISA) kit (ELISA, R&D Systems Inc., Minneapolis, MN, USA) according to manufacturer procedures, and results were raised as picogram per milliliter.

2.4. Assay of NO production

NO production was evaluated by the Griess reaction. Cell culture supernatants were mixed with an equal volume of Griess reagent [1% (w/v) sulfanilamide and 0.1% (w/v) N-(1-naphthyl)ethylenediamine in 5% (v/v) phosphoric acid, Sigma-Aldrich] in a 96-well plate and incubated at room temperature for 10 min. Absorbance was measured at 550 nm on a microplate reader (Bio-Rad Labs). NO concentration was determined from a standard curve of serial concentrations of sodium nitrite (10–100 μM).

2.5. Immunofluorescence imaging

BV-2 cells were fixed in 4% paraformaldehyde for 20 min and blocked with 10% goat serum in PBS. The glass slide with cells were incubated overnight in a humidified chamber at 4 °C with the following primary antibody (ionized calcium-binding adapter molecule-1, Iba-1, 1:200, rabbit polyclonal, Abcam, Cambridge, MA, USA). After primary antibody incubation, slide were washed and incubated with the appropriate fluorescent-conjugated secondary antibody (1:500 dilution, Sigma-Aldrich) for 1 h. Images were captured with a Nikon TE2000U microscope. The intensity of Iba-1 signal of each nucleus was counted at a magnification of 200× in at least six separate experiments by using Image-Pro Plus 6.0 software.

2.6. Conditioned medium (CM)

Microglia are considered to play an important role in bystander neuronal survival. To obtain CM, BV-2 cells were seeded in 60 mm culture plates at a density of 3 × 10^5 cells/plate. After the cells became confluent, they were incubated with serum-free DMEM for 1 h. Cells were transferred to fresh serum-free DMEM and exposed to hypoxia for 24 h in the presence or absence of resveratrol. Controls received vehicle or resveratrol. After 24 h, this medium were collected and stored at −80 °C till further used.

2.7. Neuronal culture and apoptosis analysis

Primary cultures of dissociated cortical cells were prepared and maintained as previously described [16]. Briefly, the cortex of postnatal day 1 mouse were dissociated and plated onto twelve-well plates pre-coated with poly-L-lysine in serum-free neurobasal medium with a B27 supplement system (Invitrogen Corp., Carlsbad, CA, USA). Cells were allowed to differentiate for 7 days, and then the neuronal medium was removed and replaced by a CM from microglial cells. Neurons were incubated with microglia-CM for 24 h, and then analyzed by MTT and tunnel staining. Controls received neuronal medium.

**Fig. 2.** Effects of resveratrol treatment on hypoxia-induced microglial activation. (A) BV-2 cells and primary microglia were exposure to hypoxia in the absence or presence of resveratrol (10 nM) for 24 h, then stained with anti-Iba-1 (red), and counterstained with DAPI (blue). Scale bar = 50 μm. Images are representative of triplicate sets. (B) Quantification of the Iba-1 was determined by a fluorescence plate reader. Values were expressed relative to the fluorescence signal of respective controls. Values represent the mean ± SD of three independent experiments. ***p < 0.001 Hy vs Con; ###p < 0.001 Hy + Res vs Hy.
Neuronal apoptosis was determined by TUNEL assay using an in situ cell death detection kit (FITC) following the manufacturer’s instructions (Chemicon, Temecula, CA, USA) as described previously [18]. Moreover, cells were counterstained by 4′, 6-Diamidino-2-phenylindole dihydrochloride (DAPI, Sigma-Aldrich). Images of TUNEL-positive cells (with a green fluorescent nucleus) were captured with a fluorescence microscope.

Fig. 3. Effects of resveratrol on hypoxia-induced pro-inflammatory cytokine mRNA expression and secretion. (A) BV-2 cells exposed to hypoxia were incubated in the absence or presence of resveratrol (1–100 nM) for 24 h. The levels of pro-inflammatory cytokines were measured by ELISA. BV-2 cells exposed to hypoxia were incubated in the absence or presence of resveratrol (10 nM) for 4 h. The relative expression levels of IL-1β (B) and TNF-α (C) gene were analyzed by Real-time RT-PCR. Each value was normalized to β-actin. Values represent the mean ± SD of three independent experiments. *p < 0.05, **p < 0.01 Hy vs Con; #p < 0.05, ##p < 0.01, Hy + Res vs Hy.

Fig. 4. Effects of resveratrol on hypoxia-induced NO production and iNOS expression. (A) NO was determined by Griess reagent after BV-2 cell exposure to hypoxia was incubated in the absence or presence of resveratrol (1–100 nM) for 24 h. (B) BV-2 cell exposure to hypoxia was incubated in the absence or presence of resveratrol (10 nM) for 24 h. The relative expression level of iNOS was analyzed by Western blot analysis. Each value was normalized to β-actin. Bar graphs showing quantification of expression levels of iNOS were determined by the Image-Pro Plus 6.0. Values represent the mean ± SD of three independent experiments. ***p < 0.01 Hy vs Con; #p < 0.05, ##p < 0.01 Hy + Res vs Hy.
microscope (IX71; Olympus, Tokyo, Japan) and counted in 10 random fields from each experimental condition. The proportion of TUNEL-positive cells was expressed as the percent of the total cells counted.

2.8. Reverse transcription-polymerase chain reaction (RT–PCR)

Total RNA was extracted from BV-2 cells using the Trizol reagent (Gibco, Invitrogen) according to the manufacturer’s instructions. RNA

![Image of Fig. 5: Effects of resveratrol on hypoxia-induced phosphorylation of MAPK in microglial cells. BV-2 cell exposure to hypoxia was incubated in the absence or presence of resveratrol (10 nM) (A) or resveratrol (1–100 nM) for 60 min and total protein was subjected to Western blot analysis using antibodies against phospho- or total forms of three MAPKs. Bar graphs showing quantification of expression levels of phospho-MAPKs/MAPKs were determined by the Image-Pro Plus 6.0. Values represent the mean ± SD of four independent experiments. *p < 0.05, **p < 0.01 Hy vs Con; #p < 0.05, ##p < 0.01 Hy + Res vs Hy.]

![Image of Fig. 6: Effects of resveratrol on hypoxia-induced IκB-α degradation and NF-κB activation. BV-2 cell exposure to hypoxia was incubated in the absence or presence of resveratrol (10 nM) (A) or resveratrol (1–100 nM) for 60 min and total protein was subjected to Western blot analysis using antibodies against IκB-α, phospho-or total forms of NF-κB p65. Levels of β-actin were used to evaluate protein loading. Bar graphs showing quantification of expression levels of phospho-NF-κB (p-NF-κB)/NF-κB and IκB-α/β-actin were determined by the Image-Pro Plus 6.0. Values represent the mean ± SD of four independent experiments. ***p < 0.001, ****p < 0.001 Hy vs Con; #p < 0.05, Hy + Res vs Hy.]
The effect of resveratrol on the morphology of microglia, cells that lead to morphological and functional changes. Therefore, to investigate whether the effect of resveratrol on the morphology of microglia, cells subjected to hypoxia were treated with or without resveratrol for 24 h. As shown in Fig. 2A, control BV-2 cells are mostly round with bright refringence and small dark nuclei; whereas, hypoxia induced an activated state presenting a rod-like bipolar or multipolar morphology with elongated cell bodies, also showed ameboid. Resveratrol (10 nM) had no significant effect on microglial morphology induced by hypoxia (Fig. 1C). Resveratrol alone also had no significant effect on microglial cell and astrocyte morphology. Similar findings were obtained in the primary microglia (Fig. 1C).

It had been reported previously that microglial activation is associated with a marked increase in Iba-1 expression. In our experiments, immunofluorescence analysis showed that at 24 h after hypoxia, the expression of Iba-1 was clearly increased. Treatment with resveratrol (10 nM) attenuated this hypoxia-induced upregulation of Iba-1 in BV-2 cells and primary microglia (Fig. 2).

3.3. Resveratrol suppresses TNF-alpha and IL-1beta production in hypoxia-stimulated BV-2 microglial cells

Microglia-mediated neuroinflammation occurs primarily due to the excessive pro-inflammatory mediators and cytotoxic factors released from activated microglia and their downstream signaling cascades. To assess whether resveratrol could suppress production of hypoxia-induced pro-inflammatory cytokines including IL-1beta and TNF-alpha, BV-2 cells exposed to hypoxia were treated with or without resveratrol for 24 h. As shown in Fig. 3A, following hypoxia for 24 h, the production of IL-1beta and TNF-alpha from BV-2 cells significantly increased compared to the control group. In contrast, treatment with resveratrol (10 nM) for 24 h reduced the production of TNF-alpha and IL-1beta in a concentration-dependent manner, as shown in Fig. 3B.

2.9. Western blot analysis

Protein concentration of BV-2 cells was determined using a BCA protein assay kit (Pierce Biotechnology, Inc.). A quantity of 20–40 μg of total proteins was loaded onto a 10–12% gradient polyacrylamide gel, electrophoretically transferred to a polyvinylidene fluoride membrane and probed with the following primary antibodies: phospho-nF-κB p65 (5536) antibody (1:500, Cell Signaling Tech. MA, USA), NF-κB (1:1000, Cell Signaling), inducible nitric oxide synthase (iNOS) (1:1000, Cell Signaling), phospho-P38 antibody (1:1000, Cell Signaling), P38 antibody (1:1000, Cell Signaling), phospho-JNK antibody (1:1000, Santa Cruz Biotechnology, CA, USA), JNK antibody (1:1000, Santa Cruz Biotechnology), Phospho-extracellular signal-regulated kinase (ERK) 1/2 (1:2000, Cell Signaling), ERK1/2 (1:2000; Cell Signaling), p-actin (1:2000; Sigma-Aldrich) was used as an internal control. Secondary antibodies were horseradish peroxidase conjugated to goat/mouse anti-rabbit IgG (1:8000, Sigma-Aldrich). The membranes were developed using an enhanced chemiluminescence detection system (Pierce, Rockford, IL).

2.10. Statistical analysis

Quantitative data were presented as the mean ± SD. Statistical analysis of data was performed with a one-way ANOVA using the post-hoc Tukey test for multiple comparisons of means. Differences were considered statistically significant if the p value was < 0.05.
increased. Co-incubation with various concentrations of resveratrol (10 and 100 nM) significantly abolished hypoxia-induced IL-1beta and TNF-alpha release. Moreover, resveratrol (10 nM) alone had no effect on the production of IL-1beta and TNF-alpha.

To elucidate the mechanisms responsible for the inhibitory effect of resveratrol on IL-1beta and TNF-alpha production, we next examined the mRNA levels of cytokines by Real-time RT-PCR. Consistent with the changes in the cytokine production, the hypoxia-induced mRNA levels of IL-1beta and TNF-alpha were also reduced by resveratrol (10 and 100 nM) following 4 h treatment, suggesting that resveratrol negatively regulated the production of IL-1beta and TNF-alpha at the transcriptional level in these hypoxia-stimulated microglial cells (Fig. 3B-C).

3.4. Resveratrol decreased NO production in hypoxia-stimulated microglial cells

Activated microglia are known to produce NO that is capable of causing neuronal damage. We found that after hypoxia exposure 24 h, there was significantly increased NO release from BV-2 cells. Treatment with resveratrol (10 and 100 nM) significantly inhibited hypoxia-induced NO release, and resveratrol (10 nM) alone had no effect on NO release (Fig. 4B).

Results from Western blot assays showed that resveratrol significantly suppressed hypoxia-increased iNOS expression in BV-2 cells as determined at 24 h post-hypoxia (Fig. 4B), with the maximal inhibitory effect being obtained at 10 nM (Fig. 4C). These results showed that resveratrol negatively regulated NO release through downregulation of iNOS protein expression in hypoxia-stimulated BV-2 cells.

3.5. MAPK pathway mediates the protection of resveratrol against hypoxia-induced injuries in BV-2 cells

The MAPK pathway has been shown to play vital roles in hypoxia-induced cytokine expression. In this study, we examined the influence of resveratrol on MAPK activity in BV2 cells. Hypoxia-treated BV-2 cells in the presence or absence of resveratrol for 60 min were subjected to Western blot analysis. As shown in Fig. 5A, resveratrol (10 nM) significantly suppressed hypoxia-induced phosphorylation levels of ERK1/2 and JNK MAPK, while activation of p38 MAPK induced by hypoxia was not affected by resveratrol. Furthermore, in another experiment of dose curve for hypoxia-induced MAPK phosphorylation (Fig. 5B), the results demonstrated that treatments for 1, 10 and 100 nM resveratrol were all effective on the inhibition of ERK1/2 and JNK MAPK activation in hypoxia-stimulated group.

3.6. Resveratrol inhibits hypoxia-induced NF-κB activation in microglial cells

We then assessed whether these anti-inflammatory effects of resveratrol on activated BV-2 cells occurred via blockade of the NF-κB signaling pathway. Fig. 6A illustrates that stimulation with hypoxia for 60 min induced significant NF-κB phosphorylation. But this effect was markedly suppressed by resveratrol (10 nM). In addition, as shown in Fig. 6B, treatments for 1, 10 and 100 nM resveratrol were all effective on the inhibition of NF-κB activation in hypoxia-stimulated group.

Hypoxic exposure significantly decreased IκBα protein levels (Fig. 6A, B) compared with the corresponding control. However,

![Fig. 8. The effect of resveratrol on hypoxia-induced bystander neuronal death. BV-2 cell exposure to hypoxia was incubated in the absence or presence of resveratrol (10 nM) for 24 h. The supernatant fractions (conditioned medium, CM) were collected and stored at −20 °C. Primary cortical neurons were treated with neuronal medium (NM) or microglial-CM for 24 h and neuronal apoptosis was measured by MTT (A) and by TUNEL staining (B). Apoptotic nucleus was quantified in 8 random fields for each experimental condition. Scale bar = 50 μm. Images are representative of triplicate sets. Values represent the mean ± SD of three independent experiments. &p < 0.05; &&p < 0.01 CM vs NM; ***p < 0.001 Hy vs Con; ##p < 0.01, Hy + Res vs Hy.](image-url)
treatment with resveratrol significantly repressed hypoxia-induced IkBα degradation (Fig. 6A, B). The data suggest that the protection of resveratrol on hypoxia-exposed microglial cells may be associated with suppression of the NF-κB signaling pathway.

3.7. Effect of NF-κB, ERK and JNK inhibitors on release of TNF-alpha and IL-1 beta in hypoxia-stimulated microglia

To determine whether the inhibition of NF-κB and ERK/JNK MAPKs is correlated with the anti-inflammatory mechanism of resveratrol, BV-2 cells were pretreated with NF-κB inhibitor–PDTC (50 μM), ERK inhibitor–PD98059 (30 μM) and JNK inhibitor–SP600125 (25 μM) for 30 min, and then exposed to hypoxia for 24 h. As shown in Fig. 7, PDTC, PD98059 and SP600125 significantly inhibited TNF-alpha and IL-1beta in hypoxia-exposed microglia, indicating that PDTC, PD98059 and SP600125 could reduce proinflammatory mediator production, indicating the anti-inflammatory effect of resveratrol is mediated through inhibiting the NF-κB and JNK/p38 MAPK pathways.

3.8. The effect of CM of hypoxia and resveratrol on neuronal viability

Activated microglia are known to release an array of inflammatory mediators that are deleterious for surrounding neurons in the CNS [19]. To assess bystander neuronal death by factors released from microglia following resveratrol treatment, primary neuronal cells were treated with different CM mentioned above. MTT assay showed that the viability of neurons in the hypoxia-CM condition was significantly decreased, while this effect was reversed by the CM treated with hypoxia and resveratrol (Fig. 8A). In addition, the viability of neurons in the CM treated alone with resveratrol (10 nM) did not differ from those of control-CM.

The TUNEL assay further revealed that TUNEL-positive neurons in the hypoxia + resveratrol-CM condition were significantly decreased compared with those of the hypoxia-CM condition (Fig. 8B). Collectively, these results suggest that resveratrol reversed hypoxia-induced neurotoxicity by suppressing inflammatory mediators released in microglia.

3.9. The role of TNF-alpha in CM on neuronal viability

TNF-alpha, which plays an important role in regulating microglial activation, is an initiator of the apoptotic cascade via the receptor-mediated signaling associated with oligodendroglial and neuronal cell apoptosis [20]. BV-2 cells were pretreated with TNF-alpha antagonist – lenalidomide [21] (10 μM) for 30 min, and then exposed hypoxia for 24 h. Lenalidomide significantly decreased hypoxia-induced mRNA levels of TNF-alpha (Fig. 9A). Next to determine whether the inhibition of TNF-alpha contributes to microglial-CM induced neuron death, BV-2 cells were pretreated with lenalidomide (10 μM) for 30 min, and then exposed hypoxia for 24 h; neuronal cells were treated with different CM from BV-2 cells for 24 h. MTT assay showed that the viability of neurons in the hypoxia-CM condition was significantly decreased, while this effect was reversed by the CM treated with hypoxia and lenalidomide (Fig. 9B).

4. Discussion

Overactivated microglia can secrete various pro-inflammatory cytokines and neurotoxic mediators, and trigger neuronal death in hypoxic damage. In this study, we investigated the neuroprotective effects of resveratrol against hypoxia-induced neurotoxicity via its anti-inflammatory actions by in vitro systems. The results showed that administration of resveratrol protected neurons against hypoxia-induced neurotoxicity through the inhibition of microglial activation and the subsequent reduction of pro-inflammatory factor release. Further study showed that resveratrol inhibited phosphorylation of the p65 subunit of NF-κB, phosphorylation of ERK1/2 and JNK but not p38 MAPK in the hypoxia-stimulated microglia.

Studies have recently shown that resveratrol is associated with neuroprotective properties in hypoxia damage in vivo in animals and in vitro in cell lines. For example, administration of resveratrol after the induction of hypoxic–ischemic in neonatal rats significantly attenuates hypoxic–ischemic-induced brain injury and concomitantly alleviates short- and long-term behavioral impairments. The cellular damage induced by oxygen and glucose deprivation in hippocampal slice cultures or in PC 12 cells was effectively attenuated by resveratrol [22,23]. Neuroprotective effects of resveratrol can be seen not only in cultured neurons but also in microglia cultures. For example, resveratrol was found to reduce LPS-induced pro-inflammatory cytokines production in microglia [10,24]. In agreement with these previous studies, we found that administration of resveratrol significantly protected neurons against hypoxia-induced neurotoxicity through the inhibition of microglial activation. Importantly, in the study that resveratrol at lower concentration (10 and 100 nM) inhibited the production of IL-1β, TNF-α and NO by microglia in response to hypoxia, compared to other studies [10,25]. The discrepancy may be due to differences in cell origin and experimental conditions.

IL-1β and TNF-alpha have been shown to play a key role in the onset and development of the cytokine cascade during an inflammatory response, and have been shown to be produced by activated microglia [26]. Recent evidence has indicated that activated microglia induced...
neuronal death through production of IL-1beta and TNF-alpha following hypoxic injuries [1]. Consequently, we investigated whether resveratrol would prevent the production of these cytokines following hypoxia activation of BV-2 microglia. Results demonstrate an inhibition of neuroinflammation by resveratrol, as observed by suppression of IL-1beta and TNF-alpha production. Similar results were reported by other authors, who demonstrated that the release of IL-1 beta, IL-6 and TNF-alpha from activated microglial cells is suppressed by resveratrol [27, 28]. Of note, the BV-2 cells were pre-treated with TNF-alpha antagonist significantly suppressed microglia-induced neurotoxicity, indicating TNF-alpha plays an important role in the neuroprotective anti-inflammatory effect of resveratrol.

In the CNS, NO is mainly produced by the microglia and its excessive production has been shown to be toxic to adjacent neurons [29]. Stimulation of microglia has been proposed to release large quantities of NO, through the activation of iNOS [30]. These observations are supported by data demonstrating that hypoxia induced iNOS expression in microglia of neonatal rats subjected to hypoxia [31]. Dragone et al. found that resveratrol diluted LPS-induced NO production in microglia [28] and astrocytes [25]. In this study, we have demonstrated that resveratrol prevents NO production by inhibiting iNOS protein expression in activated BV-2 microglia, suggesting another mechanism for inhibiting neuroinflammation. In addition, resveratrol has long been recognized as a natural antioxidant and demonstrated the ability of resveratrol to scavenge both O2- and OH- radicals both in vivo and in vitro [32]. Of note, resveratrol is reported to be beneficial in neurological disorders by attenuating this oxidative stress. For example, in CNS, resveratrol increases the antioxidant capacity of neuronal cells and suppresses lipid peroxidation in ischemia injury [33]. Importantly, resveratrol exerts its neuroprotective actions against activated microglia by scavenging reactive oxygen species (ROS) [3]. Taken together, these data and the results of the present study suggested inhibition of microglial activation and the subsequent production of various proinflammatory factors and ROS contributed to resveratrol-mediated neuroprotection.

As an important transcription factor in the regulation of the expressions of pro-inflammatory mediators and enzymes, NF-κB can be activated by LPS, hypoxia or inflammatory cytokines. In resting cells NF-κB is complexed with its inhibitor IκB and retained in the cytoplasm. IκB can be phosphorylated by the IκB kinase, IKK. Phosphorylation of IκB leads to its ubiquitination and subsequent degradation. NF-κB is then free to enter the nucleus and affect transcription [34]. Resveratrol prevents the activation of NF-κB and thus reduces the up-regulation of pro-inflammatory cytokines, which play a critical protective role in inflammatory reactions [10,35,36]. In line with these findings, the data of our study show that inflammatory cytokine production and NF-κB activation were reduced in hypoxia-induced microglia treated with resveratrol.

MAPKs also play important roles in the up-regulation of proinflammatory gene expression, and are considered promising targets for the treatment of neuro-inflammatory diseases [37]. It has been reported that hypoxia activates ERK, p38 and JNK MAPK in rodent microglia and animal models [38,39]. The ERK, JNK, and p38 MAPKs are all activated by hypoxia and are important upstream modulators for the production of proinflammatory mediators such as TNF-alpha, IL-1 beta, IL-6 and NO [40,41]. Corroborating these findings, the present results show that increased concentrations of pro-inflammatory cytokines resulting from hypoxia are accompanied by phosphorylation of ERK, p38 and JNK MAPK. Of particular significance to the present report were the findings that resveratrol treatment prevented phosphorylation of ERK and JNK, but not p38 MAPK, and, in this way, reduces the up-regulation of pro-inflammatory cytokines. This result is inconsistent with some previous studies [10,25]. The exact reason for this discrepancy is unclear. Some possible explanations may include differences in resveratrol treatment protocols and specifics regarding the inflammatory model.

As motioned above, the NF-κB and MAPKs are activated by hypoxia and are important upstream modulators for the production of pro-inflammatory mediators. Of note, we found that resveratrol remarkably down-regulated the increase in phosphorylation levels of the p65 subunit of NF-κB, and ERK1/2 and JNK in hypoxia-exposure BV-2 cells. The BV-2 cells were pre-treated with specific inhibitors, PDTC against NF-κB, PD98059 against ERK1/2 and SP600125 against JNK MAPK and, and then the productions of TNF-alpha and IL-1beta NO was determined. It was shown that the inhibitors significantly suppressed the overproductions of the TNF-alpha and IL-1beta in the hypoxia-exposed BV-2 cells. These results strongly supported the contention that NF-κB, ERK1/2 and JNK signaling participated in the neuroprotective anti-inflammatory effect of resveratrol.

In conclusion, this investigation demonstrates that resveratrol significantly attenuates overactivation of microglial cells by repressing expression levels of neurotoxic pro-inflammatory mediators and cytokines via activation of ERK, JNK MAPK as well as NF-κB signaling pathways. These data suggest that resveratrol-based therapies have potential for use in the treatment of hypoxic brain injury.

Authors' contribution

DXL and ZW were involved in study design and data interpretation and writing of the manuscript; QZ and LY performed the majority of the laboratory work and contributed to the analysis of data; QRZ, YG, GHL, MX and XW were responsible for western blot.

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Reference
