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Resveratrol attenuates hypoxia-induced neurotoxicity through inhibiting microglial activation

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ABSTRACT

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 exerts 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.

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. They are activated in response to specific stimuli, transform from a ramified basal homeostatic phenotype to an activated phagocytic phenotype and release a host of pro-inflammatory cytokines, chemokines 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 (Aβ) or ethanol-induced toxicity in the PC12 cells [7,8]. Resveratrol suppressed alcohol-induced cognitive deficits and neuronal apoptosis [9]. In addition, resveratrol has

Abbreviations: CNS, central nervous system; CM, conditioned medium; DAPI, 4′, 6-diamidino-2-phenylindole dihydrochloride; DMEM, Dulbecco's modified Eagle medium; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; iNOS, inducible nitric oxide synthase; IL, interleukin; LPS, lipopolysaccharide; MTT, 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide sodium; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor κB; NO, nitric oxide; PBS, phosphate buffered saline; RT–PCR, reverse transcription–polymerase chain reaction; TNF-alpha, tumor necrosis factor-alpha.

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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% CO_2 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% CO_2 + 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% CO_2 [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

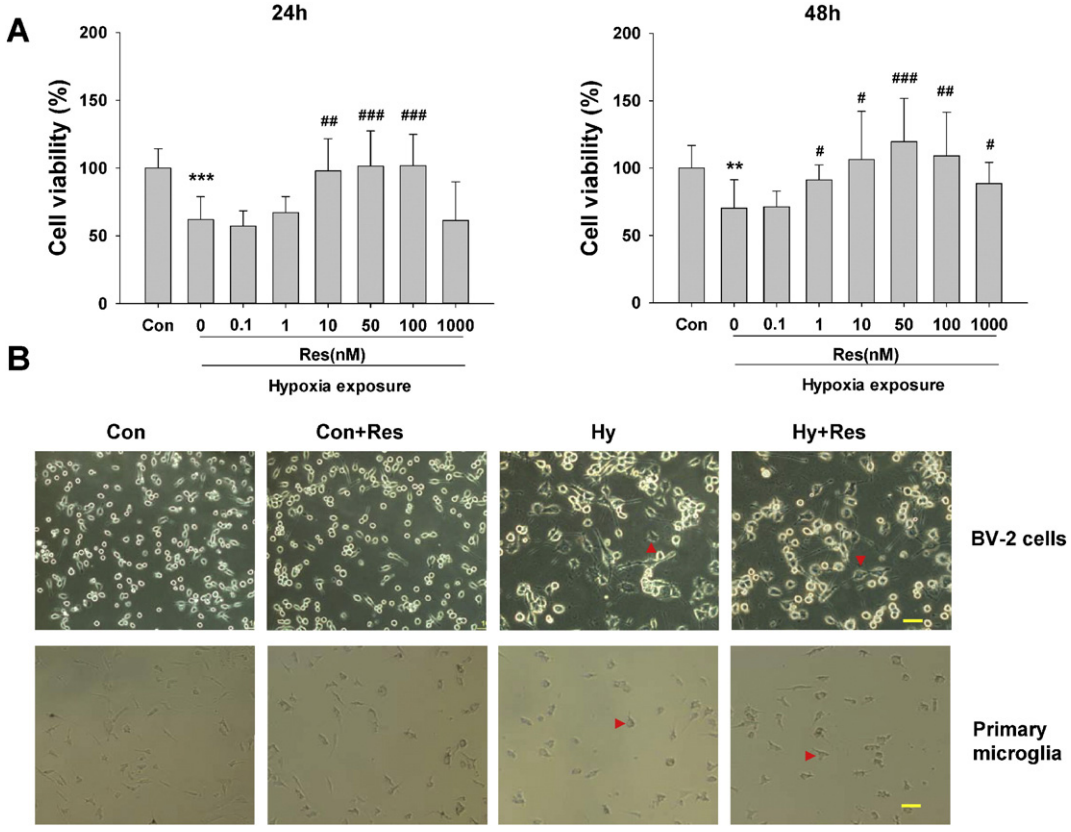

Fig. 1. Effects of resveratrol treatment on cell viability in microglia. (A) BV-2 cells maintained in serum-free medium exposure to hypoxia were incubated in the absence or presence of indicated concentrations of resveratrol (Res) (0–1000 nM) for 24 h and 48 h, and cell viability was examined by MTT 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.