Radix Scrophulariae extracts (harpagoside) suppresses hypoxia-induced microglial activation and neurotoxicity

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Abstract

Background: Hypoxia could lead to microglia activation and inflammatory mediators’ overproduction. These inflammatory molecules could amplify the neuroinflammatory process and exacerbate neuronal injury. The aim of this study is to find out whether harpagoside could reduce hypoxia-induced microglia activation.

Methods: In this study, primary microglia cells harvested from neonatal ICR mice were activated by exposure to hypoxia (1 % O₂ for 3 h). Harpagoside had been shown to be no cytotoxicity on microglia cells by MTT assay. The scavenger effect of harpagoside on hypoxia-enhanced microglial cells proliferation, associated inflammatory genes expression (COX-II, IL-1β and IL-6 genes) and NO synthesis were also examined.

Results: Hypoxia enhances active proliferation of microglial cells, while harpagoside can scavenge this effect. We find that harpagoside could scavenge hypoxia-enhanced inflammatory genes expression (COX-2, IL-1β and IL-6 genes) and NO synthesis of microglial cells. Under 3 h’ hypoxic stimulation, the nuclear contents of p65 and hypoxia inducible factor-1α (HIF-1α) significantly increase, while the cytosol IκB-α content decreases; these effects can be reversed by 1 h’s pre-incubation of 10⁻⁸ M harpagoside. Harpagoside could decrease IκB-α protein phosphorylation and inhibit p65 protein translocation from the cytosol to the nucleus, thus suppress NF-κB activation and reduce the HIF-1α generation.

Conclusion: These results suggested that the anti-inflammatory mechanism of harpagoside might be associated with the NF-κB signaling pathway. Harpagoside protect against hypoxia-induced toxicity on microglial cells through HIF-α pathway.

Keywords: Harpagoside, Hypoxia, Microglial cells, Activation, Anti-inflammation

Background

The central nervous system (CNS) consists of both neurons and non-neuronal glial cells including microglia and astrocytes; microglia, the resident macrophages of brain, play critical roles in the maintenance of neural environment [1]. Microglia cells also appear to play an important role during normal function of the nervous system. Under diverse neurological conditions, such as stroke, Alzheimer’s disease (AD), Parkinson’s disease (PD), and nerve injury-induced neuropathic pain, or exposure to toxicological stimuli, microglia will be rapidly activated [2]. Activated microglial cells display macrophage-like characteristics including production of pro-inflammatory cytokines, antigen presentation and phagocytosis [3]. However, uncontrolled and sustained secretion of inflammatory cytokines by microglia also trigger neuronal cell death [4]. Inflammatory components related to neuro-inflammation include microglia and astrocytes, the complement system, as well as cytokines and chemokines [5].

In Alzheimer’s disease (AD), it has been observed in epidemiological studies that treatment with non-steroidal anti-inflammatory drugs (NSAIDs) decreases the risk for developing AD. Unfortunately, clinical trials of NSAIDs in AD patients have not been very fruitful. Proinflammatory...
Preparations of Harpagophytum procumbens, known as devil’s claw, are used as an adjunctive therapy for the treatment of pain and osteoarthritis. Pharmacological evaluations have proven the effectiveness of this herbal drug as an anti-inflammatory and analgesic agent [7]. Harpagoside is thought to be the active principle and may represent potential anti-inflammatory drugs [8]. In this study, the anti-inflammatory activities of the main component of aqueous extracts from the Radix Scrophulariae, harpagoside, was evaluated in vitro to investigate their suppressive effect on the hypoxia-induced microglial cells activation.

**Methods**

**Preparation of microglial cells culture**

This study received prior approval of the Taipei Medical University Investigation Research Board Committee. Enriched microglial cells culture were derived from the ICR mice neonatal cortex (3–4 days old) using a technique modified from Saura et al. that favors the survival and proliferation of glial cells over neurons [9]. Briefly, mixed glial cultures were prepared from mechanical and chemical dissociation, cortical cells were seeded in DMEM (Life Technologies Inc., Gibco/BRL Division, Grand Island, New York, USA) with 10 % FBS (Life Technologies Inc., Gibco/BRL Division, Grand Island, New York, USA) at a density of 250,000 cells/ml (=62,500 cells/cm²). Cells were cultured at 37 °C in humidified 5 % CO₂/95 % air. Medium was changed every 3 days and influence of microglial cells culture was achieved after 10–14 days in vitro. Then, microglial cells culture was prepared by mild trypsinization (0.05 % trypsin + 0.2 mM EDTA) and shaking. High purity of microglia can be isolated by shaking off loosely adherent cells (astrocytes) from mixed glial cultures. For the following study, the microglial cells were grown in DMEM supplemented with 10 % fetal bovine serum. The surgical procedures were carried out in accordance with the Declaration of Helsinki and experimental protocols were approved and under supervision by the Medical College’s Animal Research Committee of the Taipei Medical University.

**The effects of harpagoside under hypoxic conditions**

The incubation under hypoxic (1 % O₂) or normoxic conditions was performed with CO₂/Tri-gas incubator (ATC-SMA-30D, Astec, Osaka, Japan). To evaluate the effects of harpagoside (Biotic Chemical, Lu Chou, New Taipei City, Taiman) under hypoxic conditions, the microglial cells were pre-incubated with harpagoside for 1 h before hypoxic test.

**3-[4,5-dimethylthiazol]-2,5-diphenylterazolium bromide assay (MTT assay)**

Microglial cells (1 × 10⁴ cells/well) were seeded into eight 96-well plates. After 2 days of incubation, harpagoside at concentrations ranging from 10⁻⁵ M to 10⁻⁹ M was added. The 3-(4, 5-dimethylthiazolyl)-2)-2, 5-diphenyltetrazolium bromide (MTT; Sigma Co., St. Louis, MO, USA) assay for cell viability was performed at the 1ˢᵗ, 3ʳᵈ, 7ᵗʰ day of culture. During the experiment, the treatment (including medium and medication) was changed every 3 days and fresh harpagoside was added at each media change. The level of mitochondrial activity of the microglial cells after harpagoside treatments were determined by colorimetric assay, which detects the conversion of MTT to insoluble formazan. The plates were read on the ELISA reader (Spectra max 340, molecular Devices; CA, USA) at a wavelength of 595 nm.

**RNA extraction, cDNA synthesis, reverse transcriptase polymerase chain reaction (RT-PCR) and quantitative real-time PCR**

After treatment, RNA for analysis was isolated at different time points. Briefly, the cell cultures were washed with PBS, total RNA was extracted and then cDNA reversely transcribed. PCR amplification was performed by the Light Cycler FastStart DNA Master SYBR Green I (Roche, Mannheim, Germany). The amplification was performed in a Roche Light Cycler 2.0 instrument under the following condition: initial denaturation at 95 °C for 10 min, 45 cycles of denaturation at 95 °C for 5 s, annealing at 55 °C for 5 s, and extension at 72 °C for 8 s. For each genes analysis, the experiments were repeated at four times. The internal standard gene used was α-tubulin and the analyzed genes were listed in Table 1.

**Measurement of NO production**

Nitrite production in the culture medium was measured of NO production using nitrate/nitrite colorimetric assay kit by the Griess reaction. Briefly, 30 μl culture medium was incubated with 15 μl of 1 % sulfanilamide (Sigma Chemical, St. Louis, MO, USA) in 5 % phosphoric acid for 5 min incubation at 37 °C, then added 15 μl of 0.1 % N-1-naphthyl-ethylenediamine dihydrochloride (Sigma Chemical, St. Louis, MO, USA) in 5 % phosphoric acid. After a 10 min incubation period at 37 °C, absorbance
was measured at 550 nm at 37 °C against a blank prepared with 60 μl of distilled water [10].

**Western blot analysis**

After treatments, culture medium was discarded and cells were lysed with Laemmli buffer (4 % SDS, 20 % glycerol, 10 % 2-mercaptoethanol, 0.004 % bromophenol blue, 0.125 M Tris/HCl, pH 6.8). Lysates were boiled for 3 min, resolved by 10 % SDS/PAGE using Tris-glycine pH 8.3 (25 mM Tris, 192 mM glycine, 0.1 % SDS) as running buffer, and then electroblotted onto a Hybond nitrocellulose membrane (transfer buffer: 25 mM Tris, 192 mM glycine, 0.1 % SDS) as running buffer. The pre-incubation of 10 μM harpagoside, hypoxia-enhanced active proliferation of microglial cells was scavenged (n = 4; Fig. 1). With 1 h pre-incubation with harpagoside, hypoxia-enhanced active proliferation of microglial cells was scavenged (n = 4; Fig. 1).

**Harpagoside scavenge hypoxia-enhanced NO synthesis of microglial cells**

Three hours' hypoxia stimulates microglial cells to up-regulate their cyclooxygenase-2 (COX-2), interleukins (IL-1β and IL-6) genes expression, with its maximal effect occurred at 6 h culture; while for the tumor necrosis factor (TNF-α) gene, its maximal effect was at 0 h culture. The pre-incubation of 10^{-8} M harpagoside again can inhibit these effects (Figs. 2 and 3).

**Harpagoside scavenge hypoxia-enhanced microglial cells active proliferation**

Under hypoxia condition (1 % O_2), active proliferation of microglial cells was observed when compared with that of normoxic control (n = 4; Fig. 1). With 1 h pre-incubation with harpagoside, hypoxia-enhanced active proliferation of microglial cells was scavenged (n = 4; Fig. 1).

**Harpagoside scavenge hypoxia-enhanced microglial cells activation**

Three hours' hypoxia stimulates microglial cells to up-regulate their cyclooxygenase-2 (COX-2), interleukins (IL-1β and IL-6) genes expression, with its maximal effect occurred at 6 h culture; while for the tumor necrosis factor (TNF-α) gene, its maximal effect was at 0 h culture. The pre-incubation of 10^{-8} M harpagoside again can inhibit these effects (Figs. 2 and 3).

**Harpagoside scavenge hypoxia-enhanced NO synthesis of microglial cells**

Three hours' hypoxia stimulates microglial cells to induce nitric oxide synthase (iNOS) gene expression; iNOS gene expression significantly upregulated with its maximal effect occurred at 6 h culture (up to 23.9 folds of the control). The pre-incubation of harpagoside can down-regulate hypoxia-induced iNOS gene expression with its maximal effect occurred at 24 h culture (down to 0.1 folds of the control (Fig. 4). Besides, we found that when microglial cells cultured under hypoxic condition (1 % O_2), active synthesis of NO was observed, this attained its maximal effect at 24 h culture (up to 127.2 % of the control). While 1 h pre-incubation with harpagoside on microglial cells does reduce the NO production by microglial cells under hypoxic condition, which attained its maximal effect at 24 h culture (down to 85.5 % of the control) (n = 4; Fig. 4).

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**Table 1 Primers sequences for reverse transcription–polymerase chain reaction (RT–PCR)**

| Gene name          | Primer                | Product (bp) |
|--------------------|-----------------------|--------------|
| IL-1β (NM8361)     | F: 5′-GTTGTGACTCTCCCTATTAG-3′ | 101          |
| IL-6 (X54542)      | F: 5′-AAGTGCATCTGTTGCTCAT-3′ | 101          |
| TNF-α (NM013693.1) | F: 5′-TCTTACTTTGCGCTCTTTTT-3′ | 150          |
| COX-2 (NM011198)   | F: 5′-TGAGGGCAATTACAGTCAGG-3′ | 94           |
| iNOS (NM010927)    | F: 5′-AATCTCAGCTCGGCACTCA-3′ | 94           |
| β-mioglobulin      | F: 5′-TCAGTGGAAGCGGACTGACTGCT-3′ | 153          |

experiments and statistically analyzed by Two-way ANOVA. Statistical significance by Dunnett's test was set at p < 0.05 between the means of the control and test groups.
Effect of harpagoside on microglial cells viability and scavenger effect on hypoxia-enhanced microglial cells activation. From this study, harpagoside has no significant cytotoxicity on microglial cells; while there is significant difference observed between treated cells ($10^{-5}$, $10^{-6}$, $10^{-7}$, $10^{-8}$, and $10^{-9}$ M harpagoside) with that of the control ($n = 4$; $p < 0.05$) at 1, 3, and 7 days' culture. We chose $10^{-8}$ M harpagoside for the further evaluation because there was maximal viability of microglial cells at this concentration (112.9 % of control, $p < 0.01$) at the 1st day's culture. Under hypoxia condition (1 % $O_2$), active proliferation of microglial cells was observed when compared with that of normoxic control ($n = 4$; $p < 0.05$). With 1 h pre-incubation with harpagoside, hypoxia-enhanced active proliferation of microglial cells was scavenged ($n = 4$). Similar to previous results, we chose $10^{-8}$ M harpagoside for the further evaluation because there was persistent effect throughout the 1st day's culture.

**Fig. 2** Effect of harpagoside on hypoxia-induced inflammatory gene expression (TNF-α, COX-2) of microglial cells. Three hours' hypoxia stimulates microglial cells TNF-α and COX-2 genes expression. The maximal effect on COX-2 gene occurred at 6 h' culture; while for the TNF-α gene, its maximal effect was at 0 h' culture. The pre-incubation of $10^{-8}$ M harpagoside again can inhibit these effects ($n = 4$). Note: *: $p < 0.05$
Harpagoside suppress hypoxia-induced microglial cells activation

Under 3 h’ hypoxic stimulation, the nuclear content of transcription factor p65 (RELA) (p65) and hypoxia-inducible factors (HIF-α) significantly increased; while the cytosol IκB-α content significantly decreased and the cytosol HIF-α content remained stationary. These effects can be reversed by 1 h’s pre-incubation of 10⁻⁸ M harpagoside (Fig. 5).

Discussion

During cerebral ischemia, exposure to hypoxia induced microglia activation; hypoxia may not only directly damage neurons, but also promote neuronal injury indirectly via microglia activation. Thus, toxic inflammatory mediators produced by activated microglia may exacerbate neuronal injury following cerebral ischemia [12]. An important factor in the onset of inflammatory process is
the overexpression of interleukin (IL)-1, which may cause dysfunction and neuronal death in a vicious circle. Other important cytokines are IL-6 and tumor necrosis factor (TNF)-α. Thus, therapeutic strategies directed at controlling the activation of microglia and astrocytes and the excessive production of pro-inflammatory and pro-oxidant factors may be valuable to control neurodegeneration in AD and dementia [13].

TNF-α plays a central role in the cytokine cascade during an inflammatory response. Neuronal production of TNF-α has also been demonstrated [14]. In the central nervous system (CNS), tumor necrosis factor-alpha (TNF-α) plays a critical role as an inflammatory mediator. There exist a positive feedback loop in the activation of microglia via TNF-alpha; this may be involved in the prolonged activation of microglia [15]. Inflammation induced by the generation of prostanoids may well contribute to neuronal destruction. Although COX-2 expression is driven by physiological synaptic activity [16] and therefore may be regarded as physiologically expressed protein in a subclass of neurons; in this study, we demonstrated that 3 h’ hypoxia stimulates microglial cells to up-regulate their TNF-α and COX-2 genes expression; while the pre-incubation of 10^{-8} M harpagoside again can scavenge these effects (Fig. 2).

Pharmacological evaluations have proven the effectiveness of harpagoside as an anti-inflammatory and analgesic agent. It inhibit the cellular expression of cyclooxygenase-2 and inducible nitric oxide by suppression of NF-kappaB activation, thereby inhibit downstream inflammation and subsequent pain events [7, 17]. IL-1 is an important initiator of the immune response, playing a key role in the onset and development of a complex hormonal and cellular inflammatory cascade. Elevated IL-1β has been detected in
the CSF and brain parenchyma within the early hours after brain injury in both humans and rodents [18]. IL-1 has also been documented to play a role in neuronal degeneration. In astrocytes, IL-1 induces IL-6 production, stimulates iNOS activity [19], and induces the production of macrophage colony-stimulating factor (M-CSF). In addition, IL-1 enhances microglial activation and additional IL-1 production, astrocyte activation, thereby establishing a self-propagating cycle [20]. IL-6 is a multifunctional cytokine that plays an important role in host defense, with major regulatory effects upon the inflammatory response [21]. IL-6 belongs to the neuropeptid family of cytokines, and it has both direct and indirect neurotrophic effects on neurons [22]. IL-6 promotes astroglisis, activates microglia, and stimulates the production of acute phase proteins [23]. In this study, 3 h hypoxia stimulates microglial cells to up-regulate their IL-1β and IL-6 genes expression, with its maximal effect occurred at 6 h culture. The pre-incubation of 10−8 M harpagoside again can inhibit these effects (Fig. 3).

Nitric oxide (NO) is a molecule with pleiotropic effects in different tissues. NO is a well-known vasorelaxant agent, but it works as a neurotransmitter when produced by neurons and is also involved in defense functions when it is produced by immune and glial cells [24]. Glial- and neuronal-derived iNOS-related long-term NO release and NO-dependent peroxynitrite formation have been demonstrated to cause neuronal dysfunction and cell death in vitro and in vivo [25–27]. Under pathophysiological conditions, NO has damaging effects. In disorders involving oxidative stress, NO increases cell damage through the formation of highly reactive peroxynitrite [28]. In this study, 3 h hypoxia stimulates microglial cells iNOS gene expression and active synthesis of NO; the pre-incubation of harpagoside can down-regulate hypoxia-induced iNOS gene expression and reduces the NO production by microglial cells under hypoxic condition (Fig. 4).

Hypoxia could lead to the inflammatory activation of microglia [29]. During cerebral ischemia, hypoxia may not only directly damage neurons, but also promote neuronal injury indirectly via microglia activation [30]. The hypoxia-inducible factor-1 (HIF-1) is primarily involved in the sensing and adapting of cells to changes in the O2 level, which is regulated by many physiological functions. The nuclear factor (NF)-kappaB transcriptional system is a major effector pathway involved in inflammation and innate immune responses. The flavonoid harpagoside is found in various herbal extracts and has shown anti-inflammatory properties. In our study, under 3 h hypoxic stimulation, the nuclear HIF-α presentation and the p65 content within nucleus significantly increased; while the cytosol IκB-α content significantly decreased; theses effect can be reversed by 1 h’s pre-incubation of 10−8 M harpagoside (Fig. 5). We report that harpagoside significantly blocks hypoxia-induced I-kappa-B phosphorylation/degradation, and NF-kappa-B transcriptional activity in mice microglial cells. Modulation of innate immunity by natural plant products may represent an attractive strategy to prevent inflammation associated with neuron dysfunction mediated by activated microglia [31].

Flavonoids and their polymers constitute a large class of food constituents, many of which alter metabolic processes and have a positive impact on health [32]. Flavonoids are a subclass of polyphenols. Microglia are innate immune cells in the central nervous system. Activated microglia can produce various proinflammatory cytokines and nitric oxide (NO), which may exert neurotoxic effects. To search for the novel therapeutic agents against neuroinflammatory diseases, we have screened a series of flavonoid compounds using a cell-based assay. Our studies showed that harpagoside appear to down-regulate the gene expression of TNF-α, interleukin (IL)-1 beta, cyclooxygenase (COX-2) and inducible nitric oxide synthase (iNOS) at mRNA levels and also may act to inhibit proinflammatory responses [31, 33]. Harpagoside also significantly suppressed 1 kappa B degradation, nuclear translocation of NF-kappa B, p65 and HIF-1α. These results indicate that harpagoside has a strong anti-inflammatory activity in brain microglia, and could be a potential therapeutic agent for the treatment of neuroinflammatory diseases [33].

Although flavonoid-rich diets and flavonoid administration prevent cognitive impairment associated with inflammation in animal studies [34], dietary bioactives have potential to restore the population of microglial cells in the senescent brain to a more quiescent state because neuroinflammation and cognitive deficits are co-morbid factors in many chronic inflammatory diseases [35]. However, retrospective cohort studies are inconsistent in showing an inverse association between dietary flavonoid intake and dementia or neurodegenerative disease risk in humans [36]. Thus, future human studies (ideally randomized clinical trials) will be required. These studies should involve supplementation with relatively high doses of specific purified flavanoids to shed light to the apparent inverse risk relationship with neurodegenerative diseases and also to determine if such compounds are therapeutically beneficial.

**Conclusions**

Hypoxia occurs when oxygen availability drops below the levels necessary to maintain normal rates of metabolism. Because of its high metabolic activity, the brain is highly sensitive to hypoxia. Hypoxia could lead to microglia activation and toxic inflammatory mediators’ overproduction. Those inflammatory molecules could amplify the neuroinflammatory process and exacerbate neuronal injury. In this study, we found that harpagoside could suppress the
CNS disorders associated with hypoxia [37]. However, the mechanisms of harpagoside have not been completely understood and the facts need to be validated by further studies both in vitro and in vivo.

Competing interests
The authors declare that there is no benefits in any form has been or will be received from a commercial party related directly or indirectly to the subject of this manuscript.

Authors’ contributions
Y-WH: (1) The conception and design of the study, or acquisition of data, or analysis and interpretation of data. Y-YS: (1) The conception and design of the study, analysis and interpretation of data; (2) drafting the article or revising it critically for important intellectual content; (3) final approval of the version to be submitted. J-SS: (1) The conception and design of the study, or acquisition of data, or analysis and interpretation of data; (2) drafting the article or revising it critically for important intellectual content; (3) final approval of the version to be submitted. M-HL: (1) The conception and design of the study. All authors read and approved the final manuscript.

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