Honokiol inhibits the inflammatory reaction during cerebral ischemia reperfusion by suppressing NF-κB activation and cytokine production of glial cells

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HIGHLIGHTS

► Honokiol inhibits brain edema and BBB injury.
► Honokiol suppresses NF-κB activation.
► Honokiol reduces NO production in cultured glial cells.
► Honokiol inhibits TNF-α production in cultured glial cells.
► Honokiol decreases RANTES production in cultured glial cells.

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ABSTRACT

This study was designed to investigate the effects of honokiol, a neuroprotective agent, on cerebral edema in cerebral ischemia reperfusion (IR) mice and its mechanism of anti-inflammation. Honokiol (0.7–70 μg/kg) significantly reduced brain water contents and decreased the exudation of Evans blue dye from brain capillaries in cerebral IR mice. Honokiol (0.1–10 μM) significantly reduced the p65 subunit level of NF-κB in the nucleus of primary culture-microglia. It (0.01–10 μM) evidently reduced nitric oxide (NO) level in the microglia culture medium and in the microglia and astrocytes coculture medium. Honokiol (0.01–10 μM) significantly decreased the level of TNF-α in the microglia medium or coculture cell medium. Honokiol (10 μM) decreased the level of Regulated upon Activation Normal T-cell Expressed and Secreted (RANTES/CCL5) protein in medium of microglia or astrocytes. In conclusion, Honokiol has a potent anti-inflammatory effect in cerebral ischemia-reperfusion mice and this effect might be attributed to its inhibition ability on the NF-κB activation, consequently blocking the production of inflammatory factors including: NO, tumor necrosis factor-α (TNF-α) and RANTES/CCL5 in glial cells. These results provide evidence for the anti-inflammatory effect of honokiol for the potential treatment of ischemic stroke.

1. Introduction

Inflammation is an important contributing factor to cerebral ischemia injury [17], and it can damage the integrity of BBB. Anti-inflammatory therapy can reduce tissue edema and improve outcome in stroke models [9]. Microglia, astrocytes and neurons are active contributors to the inflammation in ischemic stroke [2]; glial cells potentiate damage to CNS cells by releasing pro-inflammatory cytokines or molecules [4]. Nuclear factor-κB (NF-κB) plays a key role in the initiation of inflammation, activated

NF-κB regulates molecules associated with inflammation, such as inducible nitric oxide synthase (iNOS) and tumor necrosis factor-α (TNF-α) [11]; inflammatory cytokines, lipopolysaccharide (LPS), and oxidized low density lipoprotein (ox-LDL) can activate NF-κB in turn [21]. Therefore, NF-κB suppression could be an effective approach to treat inflammatory diseases. The regulation of regulated upon activation, normal T-cell expressed and secreted (RANTES/CCL5) protein is coupled to the NF-κB pathway and plays an important role in the inflammatory process [29].

Honokiol is the main biphenyl neolignan isolated from the cortex of *Magnolia officinalis* used in traditional Chinese medicine [7,23], which has various bioactivities, including anti-oxidation [6], anti-apoptosis [20] and reducing cerebral infarction [12]. Honokiol inhibits the inflammatory response in neutrophils, macrophages and lymphocytes [8]. However, the effect of honokiol on the

inflammatory response during cerebral ischemia has not been completely understood. This study investigated the influence of honokiol on the inflammatory response of cerebral ischemia-reperfusion (IR) and explored its anti-inflammatory mechanism.

2. Materials and methods

2.1. Experimental animals

SD rats born at 48 h and Kunming mice born at 6 weeks of both genders (28–33 g) were obtained from Department of Laboratory Animal Science of Peking University. The experimental procedures were approved by the Beijing Committee on Animal Care and Use.

2.2. Measurement of the exudation of cerebral capillary and brain water content in cerebral IR mice

The experiment was performed as described by Xiong and Guo [28]. The IR model was prepared by blocking bilateral carotid arteries for 30 min and then re-perfusing for 24 h after anesthetized with phenobarbital sodium. Sham operation was just separated carotid arteries. Animals were treated (i.p.) with vehicle (normal saline), honokiol or Ginaton (as positive control) at 15 min after ischemia and 15 min after reperfusion, respectively, and received Evans blue (50 mg/kg, i.v.) 1 h before sacrificed. Brains were collected and weighed, and blue dye was extracted. OD_{630} of the extracts was detected with spectrophotometer. Brains were dried at 110 °C and weighed; the brain water content (%) was calculated.

2.3. Primary cell cultures

Glial cells were prepared from rostra mesencephali and cerebral hemispheres of neonatal rats as described [25]. The coculture glial cells were separated into microglia cells and astrocytes after 10 days. Suspended microglia was collected from the medium of cell cultures as described [16]. Cells were counted and seeded in 96-well plates or 6-well plates for different assay and grown for 24 h at 37 °C in a humidified atmosphere enriched with 5% CO_2.

Astrocyte cultures were prepared and subcultured as described [17], then seeded in 96-well plates (1×10^5 cells/well) for NO or RANTES/CCL5 detection.

2.4. Drug treatment

Cells were cultured to confluent and treated with vehicle or honokiol (0.01–10 μM) for 1 h, then the medium was changed and cells were incubated with LPS (1 μg/ml) for 6–48 h to induce cell inflammation, then medium was collected for use.

2.5. Western blot analysis of nuclear translocation of p65 sub-unit of NF-κB

Microglia cells were seeded in 6-well plates with 2×10^6 cells/well, pre-treated with honokiol for 1 h, and stimulated with LPS for 24 h. Cells were collected and lysed, nuclear protein was extracted following kit instructions, separated by 12% SDS-PAGE, blotted onto PVDF membrane, and incubated with p65 mouse monoclonal antibody, then with antimouse IgG horseradish peroxidase conjugate secondary antibody. The expression of p65 was visualized using a chemiluminescent reagent (Thermo Scientific). The protein density was measured with a ChemiDoc XRS System (Bio-Rad) and analyzed as previously described [1].

2.6. Determination of NO

Cells were treated in the same way as in Section 2.4. The NO level in medium was determined by nitrites detection. The medium was mixed with fresh DAN solution at 25 °C for 20 min, then the fluorescent product was measured with excitation at 365 nm and emission at 450 nm after adding NaOH [18]. Nitrites were determined by standard curve.

2.7. Detection of TNF-α by ELISA

Glial cells were treated as in Section 2.4. After incubation, the level of TNF-α in medium was measured with rat monoclonal anti-mouse TNF-α antibody as the capture antibody and goat biotinylated polyclonal anti-mouse TNF-α antibody as the detection antibody (Rat TNF-α ELISA kit; Invitrogen) as described [31].

2.8. Detection of RANTES/CCL5 by ELISA

Glial cells were pre-treated with honokiol and LPS as in Section 2.4. The level of RANTES/CCL5 in medium was measured using a solid phase sandwich Rat ELISA kit (Invitrogen) after 24 h incubation.

2.9. Statistical analysis

All data were expressed as mean ± sd. One-way ANOVA/Dunnnet's tests were used to determine the statistical significance of differences between the treated groups and the control groups. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. The effect on brain water contents in cerebral ischemia-reperfusion mice

Cerebral IR resulted in a significant increase of brain water contents, honokiol significantly reduced the water contents and the effective dose of honokiol (0.7–70 μg/kg) is far lower than positive agent Ginaton (60 mg/kg, Table 1), thus indicating that honokiol has a more potent effect in alleviating cerebral edema than Ginaton.

3.2. The effect on Evans blue exudation from brain capillaries of cerebral IR mice

Cerebral IR resulted in a markedly increase of blue dye exudation; honokiol significantly and does-dependently decreased the amount of Evans blue (Fig. 1). The blue dye concentration after treatment with vehicle, 70 μg/kg of honokiol or 60 mg/kg of Ginaton was 34.7 ± 9.1, 19.9 ± 4.9 μg/g and 27.0 ± 6.4 μg/g, respectively.

Table 1
Effect of honokiol on brain water contents in cerebral ischemia-reperfusion in mice (± sd, $n = 10$).

Group	Dose (μg/kg)	Brain water (%)
Sham	–	80.24 ± 0.56
IR	–	81.30 ± 0.43[~]
Honokiol	0.7	80.81 ± 0.41[*]
	7	80.65 ± 0.65[*]
	70	80.39 ± 0.80[**]
Ginaton	60 mg/kg	80.65 ± 0.49[**]

Data were expressed as mean ± sd. ($n = 10$).

[*] $P < 0.05$ vs. IR group.
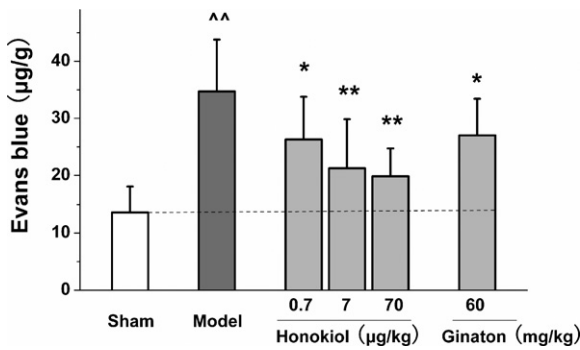[**] $P < 0.01$ vs. IR group.
[~] $P < 0.01$, vs. Sham operation group.

Fig. 1. Effect on Evans blue exudation from brain capillaries in IR mice. Data were expressed as mean ± sd, $n = 10$. $^{\frown\frown}P < 0.01$ *vs* Sham operation group; $^*P < 0.05$, $^{**}P < 0.01$ *vs* IR group.

3.3. Effect of honokiol on NF-κB pathway activation in microglia

The level of the p65 subunit of NF-κB in the nucleus represents the translocation (activation) level of NF-κB. The LPS stimulated the elevation of the p65 level in the nucleus, but honokiol significantly reduced the p65 level in a concentration dependent way. The p65 relative density for the LPS model was 1.42 ± 0.15, and it was 0.98 ± 0.15, 0.66 ± 0.04, 0.44 ± 0.09, 0.33 ± 0.03 for 0.01, 0.1, 1,10 μM of honokiol, respectively (Fig. 2).

3.4. The effect of honokiol on NO production

LPS significantly elevated the NO level in microglia medium; honokiol evidently suppressed the NO elevation. The NO level in the vehicle group and for 10 μM of honokiol treated group was 36.8 ± 7.0 μM, 13.3 ± 1.7 μM, respectively (Fig. 3A). The NO level in medium of LPS-stimulated astrocytes showed a smaller elevation than that in microglia medium and the inhibitory effect of honokiol on the NO level in microglia was mild (Fig. 3B). However, the NO level in the medium of coculture glial cells stimulated by LPS was higher than the NO level either in the medium of microglia or astrocytes, and honokiol significantly suppressed NO level in the coculture medium The NO level in the coculture medium in vehicle treated group and 10 μM honokiol treated group was 65.7 ± 7.5 μM, 30.2 ± 4.3 μM, respectively (Fig. 3C).

3.5. Effect of honokiol on TNF-α expression

LPS significantly increased TNF-α level in microglia medium, honokiol strongly reduced the level of TNF-α, and the effect lasted from 6 to 24 h. The effect of honokiol in microglia was more potent than in coculture glial cells, 0.1 μM of honokiol reduced TNF-α
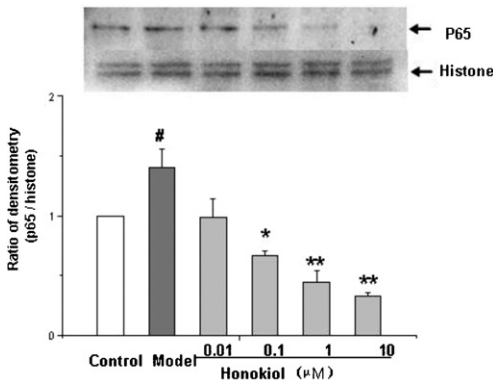

Fig. 2. Effect on LPS-induced NF-κB activation in microglia. Data were expressed as mean ± sd, ($n = 3$). $^\#P < 0.01$ *vs* the un-stimulated; $^*P < 0.05$, $^{**}P < 0.01$ *vs* LPS model.
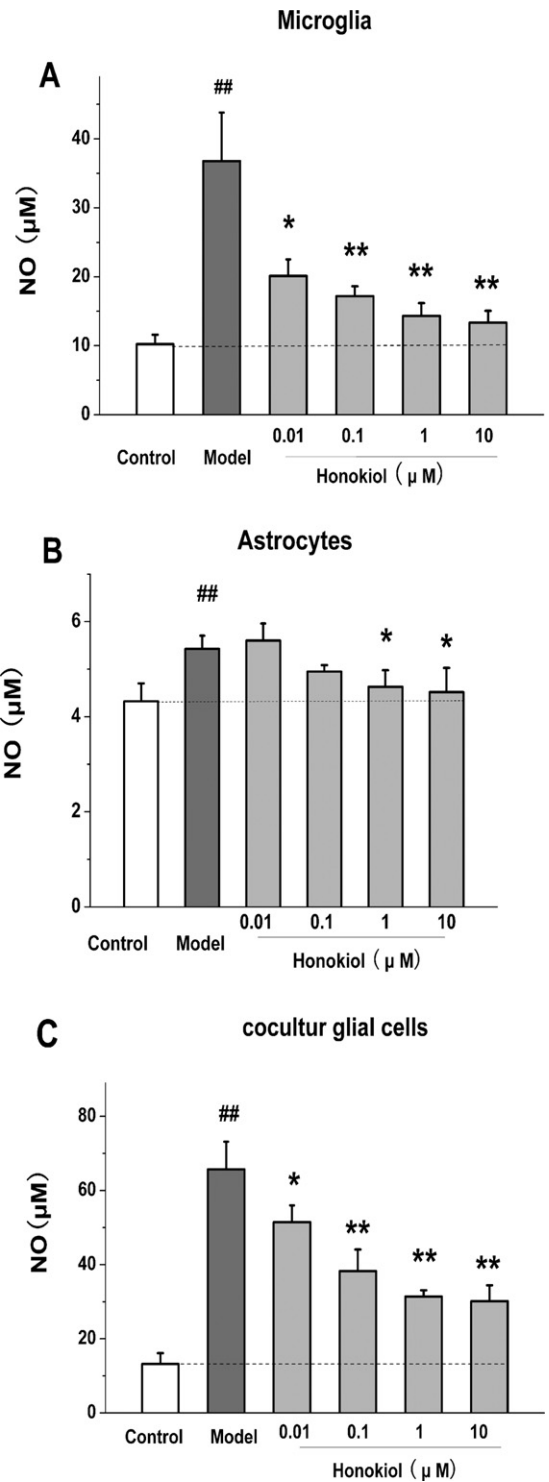

Fig. 3. Effects on NO production in LPS-stimulated microglia (A), astrocytes (B) and mixed glial cells (C), the results were expressed as nitrites concentration. The data were expressed as mean ± sd, $n = 4$. $^{\#\#}P < 0.01$ *vs* the un-stimulated; $^*P < 0.05$, $^{**}P < 0.01$ *vs* LPS model.

by 98.1% (from 1507.4 ± 37.5 to 28.9 ± 15.7 pg/ml) in microglia (Fig. 4A) and by 70.0% (from 168.4 ± 43.1 to 49.4 ± 7.1 pg/ml) in coculture glial cells (Fig. 4B) at 6 h.

3.6. Effect of honokiol on RANTES production

LPS stimulated a significant elevation of RANTES/CCL5 in glial cell medium, honokiol (10 μM) significantly reduced RANTES/CCL5
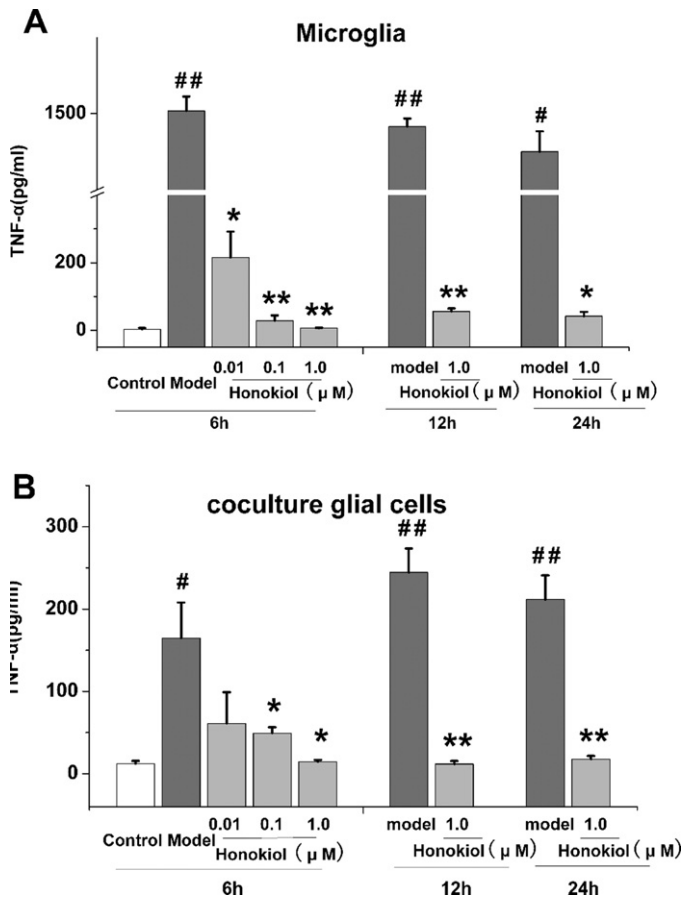
Fig. 4. Effects on TNF-α expression in LPS-stimulated rat microglia (A) and mixed glial cells (B). Data were expressed as mean ± sd, (n = 3). #P < 0.05, ##P < 0.01 vs the un-stimulated; *P < 0.05, **P < 0.01 vs model.
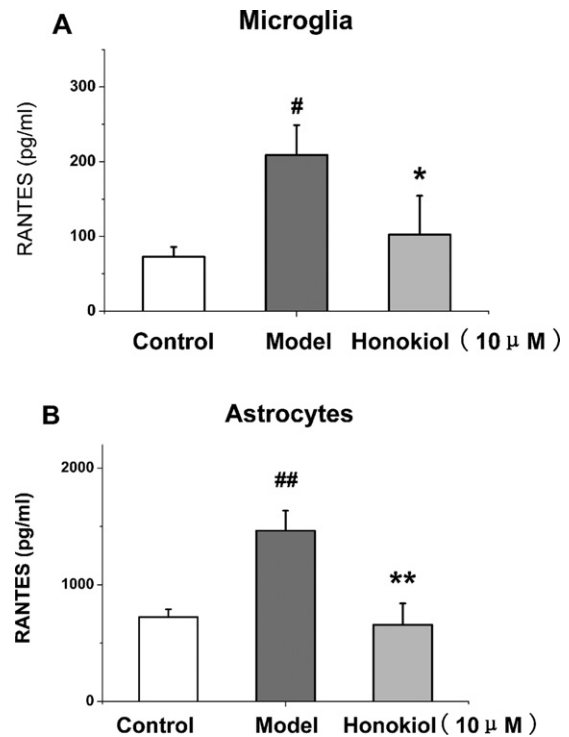

Fig. 5. Effects on LPS-induced RANTES expression in microglial cells (A) and astrocytes (B) Data were expressed as mean ± sd, n = 3. #P < 0.05, ##P < 0.01 vs the un-stimulated; *P < 0.05, **P < 0.01 vs model.

contents in medium of microglia (Fig. 5A), and the inhibitory effect on RANTES/CCL5 was more potent in astrocytes than in microglia cells (Fig. 5B).

4. Discussion

Other authors reported the anti-inflammatory effects of honokiol through inhibiting ROS in neutrophils [13], and its suppression on LPS-induced TNF-α and NO expression in macrophages [14]. This study demonstrated the potent anti-inflammatory effect of honokiol on the cerebral IR injury and the LPS-induced inflammatory response of glial cells. 70 μg/kg of honokiol reduced dye exudation by 70.6% (Fig. 1) in vivo and 10 μM of honokiol reduced NO production in coculture glial cells by 67.8% (Fig. 4C) in vitro, which is consistent with activity in vivo.

In this study, we demonstrated that honokiol significantly reduced brain water content in cerebral IR mice (Table 1). This finding indicates honokiol may alleviate brain edema. The brain blood vessel system is the structural basis of BBB and the production of cytokine potentiates the damage of BBB [22]. This study found that honokiol significantly inhibited the dye exudation during cerebral IR in mice, suggesting it may inhibit cerebrovascular inflammation therefore reducing the damage of BBB. Ginaton was used as the positive control since it is commonly used in ischemic stroke treatment in China nowadays [10,24]. Honokiol showed a more potent effect than Ginaton because its effective dose was about 1000 times lower than the latter (Table 1, Fig. 1).

To establish a cell inflammatory model, NO was used as an inflammatory marker and 1 μg/ml of LPS was used as an

inflammatory stimulator, which seemed to be an eligible concentration for inducing inflammation but failed to influence cell viability (data not shown).

Based on the fact that honokiol inhibited NF-κB activation in mouse B cells [20], HUVECs and macrophages [14], the influence of honokiol on NF-κB activation in LPS-stimulated primary glial cells was investigated and it significantly inhibited the translocation of NF-κB/p65 (Fig. 2). The results suggest that honokiol inhibited the NF-κB activation in microglia cells and consequently inhibited the overexpression of pro-inflammatory mediators.

NO is an important neurotoxic factor during cerebral ischemia. This study revealed the effects of honokiol on the NO production in microglia, astrocytes or their coculture. LPS elevated the NO level in microglia medium, but honokiol potently inhibited this elevation (Fig. 3A). In astrocytes, the NO level was lower and did not show significant elevation by LPS, and the inhibitory effect of honokiol was mild (Fig. 3B). This indicates that microglia is the principle source of NO and honokiol mainly inhibits the NO production in microglia cells. Furthermore in LPS-stimulated coculture glial cells, the NO level in the coculture medium was higher than in either microglia or in astrocyte mediums, which is likely due to the mutual regulation between astrocytes and microglias on NO production, namely, the activated microglia promote astrocyte activation [5]. Honokiol also suppressed the LPS-induced NO elevation in coculture glial cells (Fig. 3C), suggesting that its anti-inflammatory effect is partly attributed to NO inhibition in glial cells. These results are consistent with the results of honokiol on macrophages [14].

Honokiol inhibits the LPS-induced TNF-α secretion in human renal mesangial cells (HRMCs) [27] and macrophages [14]. In this study we revealed the inhibitory effect of honokiol on TNF-α in glial cells (Fig. 4A) and LPS-induced TNF-α elevation in coculture glial cells was only about 1/8 of that in microglias it is because astrocytes mainly produce TGFβ-1 which reduces microglia activation [2]. However, honokiol significantly suppressed LPS-induced TNF-α elevation in coculture glial cell medium (Fig. 4B), suggesting that

the inhibition of TNF-α may participate in its anti-inflammatory effect.

Both neurotoxic [19] and neuroprotective effects [3] of RANTES/CCL5 have been described; honokiol inhibits high-glucose-induced upregulation of RANTES/CCL5 in HRMCs [26]. In this study the inhibitory effects of honokiol on RANTES/CCL5 expression in both microglia cells (Fig. 5A) and astrocytes (Fig. 5B) was demonstrated. These data indicates that the anti-inflammatory effect of honokiol is related to the RANTES/CCL5 inhibition, and RANTES/CCL5 in our study seems to be a neurotoxic factor but a neuroprotective factor.

In conclusion, honokiol has a potent anti-inflammatory effect in cerebral IR and this effect might be attributed to its powerful inhibition on NF-κB activation, consequently blocking the production of inflammatory factors. These results provide novel evidence for the anti-inflammatory effects of honokiol for the treatment of ischemic stroke.

Acknowledgments

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