Harmine is an inflammatory inhibitor through the suppression of NF-kB signaling

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ABSTRACT

Harmine is a major constituent in a hallucinogenic botanical mixture ayahuasca and medical plant Peganum harmala L. The plant is used for various illnesses and exhibits anti-inflammatory activity. However, the active constituents remain unclear. Here, we screened the seven alkaloids in P. harmala for their anti-inflammatory activity using an nuclear factor-kB (NF-kB) reporter assay. We found that harmine and harmol could inhibit NF-kB transactivity. As the most abundant compound, harmine inhibited tumor necrosis factor-a (TNF-a)- and lipopolysaccharides (LPS)-induced NF-kB transactivity and nuclear translocation in mouse macrophage RAW 264.7 cells. The mRNA and protein levels of NF-kB downstream inflammatory cytokines also reduced. In an LPS-challenged mouse model, harmine markedly averted inflammatory damage of the lung, and decreased serum TNF-a, interleukin-1β (IL-1β) and IL-6 levels. Our data indicate that harmine may exert the anti-inflammatory effect by inhibition of the NF-kB signaling pathway and harmine is probably responsible for the anti-inflammatory effects of P. harmala.

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1. Introduction

Peganum harmala L (Zygophyllaceae) is a medical plant native to arid areas from North Africa to China [1,2]. In folk medicine, P. harmala is used for a variety of illnesses and anti-inflammatory activity has been noted [1–5]. Inflammation is a protective response to harmful stimuli, such as pathogens, to eliminate necrotic cells and damaged tissues and to initiate tissue repair. Long-term inflammation may result in the formation of chronic disease. NF-kB is a pivotal proinflammatory transcription factor, composed by heterodimeric proteins with molecular masses of 50 kDa (p50) and 65 kDa (p65) [6,7]. NF-kB is distributed in the cytoplasm in its unstimulated form and is activated by many inflammatory stimuli including LPS and TNF. Most of these agents induce phosphorylation and translocation of p65 to the nucleus, where it regulates gene expression of inflammatory cytokines [8]. NF-kB is involved in the inflammatory responses of various chronic diseases. Dysregulation of NF-kB signaling is beneficial to those with inflammatory and infectious diseases. Targeting NF-kB signaling is a strategy for the treatment of inflammatory diseases [9].

Seven alkaloids, harmaline, harmine, harmalol, harmol, vasicine, vasicinone and deoxyvasicine, are abundant in P. harmala [10]. However, the active constituents and the mechanism for the anti-inflammatory effects of P. harmala are still uncertain. In the present study, we tested the inhibitory effects of these alkaloids on NF-kB transactivity and revealed that harmine and harmol could inhibit NF-kB signaling.

Harmine is not only found in P. harmala, but is one of the major components in ayahuasca which is used as a hallucinogen [11]. Recent studies have shown that harmine exhibits many pharmacological activities, such as anti-tumor [12–14] and antimicrobial properties [15–17], and is considered for its anti-Alzheimer activity [18,19]. Here we show that harmine may be responsible for the anti-inflammatory effects of P. harmala.

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2. Materials and methods

2.1. Reagents

Harmine, harmaline, harmalol, harmol, vasicine, vasicinone and deoxyvasicine were purified from *P. harmala* as in our previous reports [10]. The purity of the compounds was >98%.

2.2. Animals

The animal protocols were approved by the Animal Care and Use Committee of Shanghai University of Traditional Chinese Medicine (approval number: 2013014). Seven-week-old ICR male mice were purchased from Shanghai Silaike Experimental Animal Co., Ltd. (Shanghai, China). All mice were housed in a controlled environment (22 °C, 12 h light/dark cycle with light period from 7 a.m. to 7 p.m.) with free access to food and water in the animal room of the Shanghai University of Traditional Chinese Medicine Experimental Center. Animals were acclimatized for 1 week before experiments. The experiments were conducted as the following groups: control group; LPS + vehicle group; LPS + dexamethasone (3 mg/kg) group; LPS + harmine (30 mg/kg) group. The mice were dosed using intraperitoneal injection in a water vehicle for 1 days and 1 h before injection of LPS. One hour after the second dose, the mice were intraperitoneally injected with LPS (Sigma, St. Louis, MO, USA) at 10 mg/kg for 6 h. Then the mice were anesthetized using 20% urethane (Sigma, St. Louis, MO, USA) and sacrificed, and the cardiac blood samples and lung tissues were collected.

2.3. Gene reporter assay

HEK-293T cells obtained from ATCC were seeded in 48-well plates and transfected with a transfection system including pCMX-NF-κB, NF-κB-Luc and Renilla-Luc plasmids and FuGENE-HD reagent (Roche, Mannheim, Germany). Subsequently, the plates were cultured overnight and then treated with the compounds for 1 h. After incubation, human recombinant TNF-α (100 ng/mL, Sigma, St. Louis, MO, USA) or LPS (1 μg/mL, Sigma, St. Louis, MO, USA) was dispensed for 12 h to stimulate the NF-κB pathway. Luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

2.4. Determination of nitric oxide (NO) production

Mouse RAW 264.7 macrophage cells were obtained from ATCC and cultured in DMEM as described previously [20,21]. The cells were seeded in 48-well plates at 37 °C overnight then treated with harmine at concentrations of 2, 5, 10, 25 and 50 μM for 1 h, and sequentially incubated with LPS (1 μg/mL) for 24 h at 37 °C. After treatment, the supernatant was collected by centrifugation of the cells. NO content was determined by a nitric oxide detection kit (Beyotime, Beijing, China) according to the manufacturer’s specifications. The absorbance was examined at 540 nm by a microplate reader. The NO production was reckoned from a sodium nitrite

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**Fig. 1. NF-κB transactivity screen of *Peganum harmala* L. constituents.** (A) Structure of harmaline, harmine, harmalol, harmol, vasicine, vasicinone and deoxyvasicine. (B) NF-κB transactivity screen. TNF-α was used to activate NF-κB. (C) Effects of harmine on LPS induced NF-κB transactivity. HEK-293T cells were co-transfected with NF-κB expression plasmids and an NF-κB-dependent reporter (NF-κB-Luc) for 24 h and treated with the compounds at the indicated concentrations for another 24 h. The relative luciferase activities were measured by comparison to Renilla luciferase activities. Data represent the mean ± SD of three independent experiments. #P < 0.05 vs. control group. *P < 0.05, **P < 0.01 vs. LPS or TNF-α group.
standard curve.

2.5. Enzyme-linked immunosorbent assay (ELISA)

RAW 264.7 cells were seeded in 48-well plates for overnight culture and then incubated with different concentrations of harmine for 1 h. Then, the cells were treated with LPS (1 μg/mL) for 24 h. The cell culture supernatants were collected to detect the cytokine content of the culture medium using ELISA according to the instructions in the kit (TNF-α, BioLegend, USA; IL-1β and IL-6, eBioscience, USA). The optical densities were examined at 450 nm and 570 nm using a microplate reader. The cytokine production in the samples was reckoned from a standard curve generated from the standard of TNF-α, IL-1β and IL-6. For the in vivo assay, the mouse blood samples were centrifuged at 800 g for 15 min. The serum was stored at −80 ºC until used.

2.6. Quantitative real-time PCR

Total RNA was extracted from cells using Trizol reagent (Takara, Dalian, China) according to our previous description [22]. Complementary DNA (cDNA) was synthesized by a reverse transcriptase kit (Fermentas, Madison, WI, USA). Quantitative real-time RT-PCR was carried out with SYBR Green QPCR Master Mix (Applied Biosystems, Waltham, MA, USA) in an ABI StepOne Plus Real-Time PCR system (Applied Biosystems, CA, USA). Primers were used as our previous described [22]. The values were normalized by the level of β-actin expression.

2.7. Immunofluorescence staining and microscopic imaging

RAW 264.7 cells were seeded onto U10 mm cover slides in 24-well plates. Different concentrations of harmine and TNF-α (100 ng/mL) were dispensed after the cells were adherent. The plates were incubated at 37 ºC for 24 h. After being rinsed with PBS thrice, the coverslips were fixed with 4% paraformaldehyde for 30 min at room temperature and then washed with PBS three times. The slides were incubated with anti-NF-κB p65 and anti-p-NF-κB p65 antibodies (Cell Signaling Technology, USA) at a 1/200 dilution for 30 min at 37 ºC and then washed with PBS twice. After that, the proteins were stained with a corresponding secondary antibody at a 1/500 dilution for 30 min at 37 ºC followed by PBS washing twice. The coverslips were counterstained for nuclei with Hoechst at a 1/10000 dilution for 5 min. After washing with PBS, the slides were prepared with mounting medium. Images were captured by a Zeiss Imager M2 microscope.

2.8. H&E staining

The lung tissues were fixed in 4% paraformaldehyde, embedded with paraffin and cut at 5 μm. The sections were stained with hematoxylin and eosin (H&E) for 7–8 min. The photos were captured by a Zeiss Imager M2 microscope.

2.9. Statistical analysis

The values are presented as the mean ± standard deviation (SD) of at least three independent experiments. SPSS program (ver. 22.0) was used for statistical analysis of the data. Statistical significance was assessed by one-way analysis of variance (ANOVA) followed by t-test. Differences were considered statistically significant at P < 0.05 for all cases.

Fig. 2. Harmine results in NF-κB nuclear translocation. (A) NF-κB p65 in RAW 264.7 cells. (B) p-NF-κB p65 in RAW 264.7 cells. The cells were treated with TNF-α plus harmine at different concentrations. RAW 264.7 cells were treated with various concentrations (2.5, 5 and 10 μM) of harmine and stimulated with TNF-α (100 ng/mL) for 30 min. Then the cells were subjected to immunofluorescence analysis as described in section 2.7. Magnification = 400×.
3. Results

3.1. Harmine significantly inhibits transcriptional activity of TNF-α-stimulated NF-κB

Seven *P. harmala* alkaloids, harmaline, harmine, harmalol, harmol, vasicine, vasicinone and deoxyvasicine (Fig. 1A), were screened for their effects on NF-κB transcription activity using a reporter assay. HEK-293T cells were co-transfected with an NF-κB expression plasmid and NF-κB-responsive Luc plasmids, and then were stimulated with TNF-α for its transactivity. Luciferase signals were captured to evaluate the anti-inflammatory effect. TNF-α significantly increased NF-κB transcriptional activity. Of the seven compounds, harmine and harmol decreased NF-κB transcription activity dramatically (Fig. 1B), while none of the other alkaloids had a marked effect on TNF-α-induced NF-κB transcriptional activity.

Since harmine is the major constituent of *P. harmala*, and easier to get than harmol, we further examined the effects of different dosages of harmine on the LPS-induced NF-κB activity (Fig. 1C). The result showed that harmine inhibited the activity in a dose-dependent manner, suggesting that harmine may be an NF-κB inhibitor.

3.2. Harmine reduces TNF-α-induced nuclear translocation of NF-κB in RAW 264.7 cells

We analyzed the effect of harmine on TNF-α-induced nuclear translocation using an immunofluorescence assay. NF-κB was distributed in the cytoplasm mostly in an untreated state. TNF-α increased the nuclear translocation of p65, and harmine suppressed TNF-α-induced NF-κB p65 nuclear accumulation at 2.5–10 μM concentrations (Fig. 2A). To confirm the effect of harmine on the suppression of translocation of NF-κB from plasma to nuclear, we then assayed its effects on p-NF-κB p65 nuclear translocation. The results showed that harmine inhibited the phosphorylation of NF-κB p65 and its nuclear translocation (Fig. 2B). These data support that harmine is an NF-κB inhibitor.

![Fig. 3. Harmine inhibits inflammatory cytokines in RAW 264.7 cells. (A) TNF-α, IL-1β and IL-6 mRNA levels in RAW 264.7 cells. β-Actin was used as an internal control for normalizing the mRNA levels. (B) TNF-α, IL-1β and IL-6 protein in culture supernatant. (C) NO production in the supernatant. The RAW 264.7 cells were treated with harmine plus 1 μg/mL of LPS for 24 h and the supernatant was collected for cytokine and NO assays. Data are presented as means ± SD (n = 4–6). #P < 0.01 vs. control group. *P < 0.05, **P < 0.01 vs. LPS group.](image-url)
3.3. Harmine suppresses proinflammatory cytokine mRNA expression in LPS-induced RAW 264.7 macrophages

To explore whether harmine suppresses NF-κB downstream signaling, the mRNA level of proinflammatory cytokines in RAW 264.7 cells was tested using quantitative real-time PCR. The results showed that the mRNA levels of TNF-α, IL-1β and IL-6 in LPS-treated cells were all significantly increased. Harmine blocked the expression of TNF-α, IL-1β and IL-6 mRNA at concentrations of 1–25 μM in a dose-dependent manner (Fig. 3A). Next, the protein levels of cytokines in the culture supernatant were analyzed using ELISA. The data showed that harmine reduced TNF-α, IL-1β and IL-6 protein levels in the supernatant at concentrations from 2 to 25 μM (Fig. 3B). These data further support that harmine suppresses NF-κB signaling.

3.4. Harmine restrains NO production in LPS-induced RAW 264.7 macrophages

Next, nitric oxide (NO), a key molecule in the regulation of inflammatory reactions [23], was evaluated to investigate the impact of harmine on LPS-induced inflammation in RAW 264.7 macrophages. The levels of NO significantly increased due to the treatment with LPS (Fig. 3C), whereas harmine pretreatment for 1 h markedly reduced the NO level in a dose-dependent manner. The data indicated that harmine may suppress the production of NO in LPS-stimulated RAW 264.7 cells.

3.5. Harmine reduces inflammation in vivo

To investigate whether harmine could inhibit inflammation in vivo, an acute lung injury mouse model was induced by intraperitoneal injection of bacterial LPS. Also known as endotoxins, LPS are a component of the surface of Gram-negative bacteria and may cause toxic effects by activating macrophages with production of inflammatory cytokines such as TNF-α, IL-1β and IL-6 through activation of the NF-κB pathway. The mice were dosed with harmine using intraperitoneal injection at 30 mg/kg in a water vehicle day and 1 h before LPS challenge. Dexamethason was used as a positive control. After 6 h of LPS injection, the mouse samples were collected. Histologic examination showed that lung tissue in the control group showed no pathological damage, with a clean alveolar cavity. However, the LPS-induced group exhibited obvious congestion, alveolar wall thickening, lumen bleeding and exudation, and some polymorphonuclear leukocyte infiltration, showing acute inflammation. Similar to the dexamethason group, the

Fig. 4. Harmine suppresses the inflammatory response induced by LPS in mice. (A) H&E staining of lung section. (B) Serum inflammatory cytokines. The mice were intraperitoneally injected with dexamethasone at 3 mg/kg or harmine at 30 mg/kg one day and 1 h before injection of LPS at 10 mg/kg. The mice were treated for 6 h, and then the blood samples and lung tissues were collected and analyzed. Data are presented as means ± SD (n = 7). #P < 0.01 vs. control group. *P < 0.05, **P < 0.01 vs. LPS group.
harmine group had notably suppressed pulmonary congestion, alveolar wall and inflammatory cell infiltration in lung tissue compared to the LPS-induced group (Fig. 4A), suggesting that harmine may prevent the inflammatory pathological changes in mice induced by LPS.

We then assayed the serum inflammatory cytokine levels in the mice. The results showed that the protein levels of TNF-α, IL-1β and IL-6 in the LPS-challenged mice were significantly higher than those in the control mice. Both dexamethasone and harmine remarkably reduced the levels of these cytokines in the mice (Fig. 4B). These data suggest that harmine may inhibit the inflammatory response in vivo via the inhibition of NF-κB signaling.

4. Discussion

In the present study, we identified that harmine and harmol are the anti-inflammatory agents from seven alkaloids in P. harmala L. We also provided evidence that the anti-infection and anti-inflammatory effects of harmine induced by LPS are likely via inhibition of the NF-κB signaling pathway.

NF-κB is a crucial regulator of inflammatory response and is involved in the pathology of many chronic diseases. Targeting the NF-κB signaling pathway could be a therapy for these diseases. Many small molecules have been identified for their anti-inflammatory effects via several different mechanisms including inhibition of protein kinases, protein phosphorylation, nuclear translocation, κB ubiquitination, p65 acetylation or induction of methylation. In the NF-κB inhibitor screen, harmine and harmol suppressed bacterial LPS- and TNF-α expression-induced NF-κB transactivity. This result is similar to a previous report showing that harmine blocks herpes simplex virus infection through down-regulation of genes involved in immune responses to infection and inflammation. NO is another inflammatory factor. Harmine significantly inhibited NO production in LPS-induced RAW 264.7 cells, further supporting the anti-inflammatory effect of harmine.

The anti-infection and anti-inflammatory effects of harmine were identified in LPS-challenged mice. LPS, also known as endotoxins, may exert acute toxic effects by activating NF-κB signaling of macrophages with production of inflammatory cytokines such as IL-1β, TNF-α and IL-6. In harmine-treated cells, both mRNA and protein levels of IL-1β, TNF-α and IL-6 were notably inhibited, which confirmed the inhibitory effects of harmine on NF-κB signaling. NO is another inflammatory factor. Harmine significantly inhibited NO production in LPS-induced RAW 264.7 cells, further supporting the anti-inflammatory effect of harmine.

In summary, we found that harmine may inhibit LPS- and TNF-α-induced NF-κB activation and p65 nuclear translocation, reduce the mRNA levels of protein genes of inflammatory cytokines IL-1β, TNF-α and IL-6 both in vivo and in vitro, and avert inflammatory damage in the lungs of mice induced by LPS. Our data suggest that harmine may be responsible for the anti-inflammatory effect of P. harmala and ayahuasca may have a potential role for inflammatory and infective diseases.

Conflicts of interest

The authors declare no conflict of interest.

References

