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The protective role of oxymatrine on neuronal cell apoptosis in the hemorrhagic rat brain

Man Huang [a], Yue-Yu Hu [a,*], Xiao-Qiao Dong [b], Qiu-Ping Xu [a], Wen-Hua Yu [b], Zu-Yong Zhang [b]

[a] Department of Intensive Care Unit, Sir Run Run Shaw Hospital, College of Medicine, Zhejiang University, 3 Qingchun East Road, Hangzhou 310000, China
[b] Department of Neurosurgery, The First Hangzhou Municipal People's Hospital, Nanjing Medical University, 261 Huansha Road, Hangzhou 310000, China

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ABSTRACT

Ethnopharmacological relevance: Oxymatrine is extracted from the traditional Chinese herb *Sophora flavescens* Ait, possesses anti-inflammatory, anti-oxidative and anti-apoptotic properties, and has been used for the treatment of chronic viral hepatitis and many other diseases.
Aims of the study: This study aimed to investigate the effects of oxymatrine on inflammatory response mediated by Toll-like receptor4 (TLR4) and nuclear factor kappa-B (NF-κB), oxidative injury induced by 12/15 lipoxygenase (12/15-LOX), phosphorylated p38 mitogen activated protein kinase (phosphor-p38 MAPK) and cytosolic phospholipase A2 (cPLA2), and neuronal cell apoptosis in rat brain with intracerebral hemorrhage (ICH).
Materials and methods: Wistar rats were treated intraperitoneally with 60 or 120 mg/kg of oxymatrine daily for 5 days following ICH. The rats were sacrificed at hour 2, 6, 12, 24, 48, 72, and 120 after ICH. The gene expressions of TLR-4 and NF-κB, the levels of TNF-alpha, interleukin-1beta, interleukin-6, 12/15-LOX, phospho-p38 MAPK and cPLA2, and the number of apoptotic neuronal cells in rat brain were determined.
Results: Oxymatrine at 120 mg/kg significantly suppressed gene expressions of TLR-4 and NF-κB, decreased levels of TNF-alpha, interleukin-1beta and interleukin-6, inhibited synthesis of 12/15-LOX, phospho-p38 MAPK and cPLA2 protein, and mitigated apoptotic neuronal changes following ICH in rat.
Conclusion: Oxymatrine at 120 mg/kg following ICH inhibits inflammatory responses, oxidative injury, and neuronal cell apoptosis in rats.

1. Introduction

Intracerebral hemorrhage (ICH) is still the leading cause of death and disability in recent years (Ribo and Grotta, 2006; Sutherland and Auer, 2006; Qureshi et al., 2009). Although the pathophysiological mechanisms of ICH are still unclear, inflammatory reaction and oxidative stress are two major factors leading to brain injuries after ICH (Wu et al., 2002; Nakamura et al., 2005; Zhang et al., 2006; Wang, 2010). Accumulating evidences have showed that Toll-like receptor4 (TLR4), nuclear factor kappa-B (NF-κB), 12/15 lipoxygenase (12/15-LOX), phosphorylated p38 mitogen activated protein kinase (phospho-p38 MAPK), and cytosolic phospholipase A2 (cPLA2) are major mediators responsible for inflammatory and oxidative brain damages after ICH (Hickenbottom et al., 1999; Zhao et al., 2007; Wagner, 2007; Nito et al., 2008;Teng et al., 2009; He et al., 2009; Cui et al., 2010a, 2010b). Thus, any interventions that inhibit TLR4, NF-κB, 12/15-LOX, phospho-p38 MAPK and cPLA2, may be expected to reduce the severity of hemorrhagic brain injury.

Oxymatrine ($C_{15}H_{24}N_2O$) (OMT) is a potent monosomic alkaloid. It is extracted from traditional Chinese herbs *S. flavescens* Ait, has been used for the treatment of chronic viral hepatitis (Zeng et al., 1999) and possesses the antiinflammatory, antioxidative and anti-apoptotic properties in many other diseases (Hong-Li et al., 2008; Zhao et al., 2008; Xu et al., 2005). Recently, several studies have demonstrated that OMT mitigates inflammatory reaction after ischemic or traumatic brain injury by inhibiting TLR4/NF-κB signaling (Fan et al., 2009; Liu et al., 2009; Dong et al., 2011), and lessens oxidative injury after ischemic brain injury by suppressing the synthesis of 12/15-LOX, phospho-p38 MAPK and cPLA2 (Cui et al., 2011). However, the exact effects of OMT on ICH remain unclear. The present study was to investigate the antiapoptotic properties of OMT and their underlying mechanisms in the hemorrhagic brain tissue.

2. Materials and methods

2.1. Study design

A total of 140 male Wistar rats (age, 8–10 weeks; weight, 250–300 g; the Animal Center of the Chinese Academy of Sciences,

* Corresponding author. Tel.: +86 0571 87065701; fax: +86 0571 86090073.
E-mail address: huyueyu2008@163.com (Y.-Y. Hu).

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Shanghai, China) were used in the study. The rats were housed in temperature- and humidity-controlled animal quarters with a 12-hr light/dark cycle. All experimental protocols strictly followed the Laboratory Animal Care and Use Guidelines of Zhejiang University.

All rats were divided into saline-treated group, sham-operation group, and two OMT-treated groups at 60 and 120 mg/kg. The OMT-treated rats were treated intraperitoneally with 60 or 120 mg/kg of oxymatrine once daily for 5 days, 0.5 h after ICH. The rats in the saline-treated and sham-operated groups received an intraperitoneal administration of 1 mL of saline 0.5 h after ICH or procedure once daily for 5 days. All animals were sacrificed by decapitation under deep pentobarbital anesthesia (80 mg/kg, intraperitoneally) at hour 2, 6, 12, 24, 48, 72, and 120 following ICH ($n=5$ at each time point from each group). Brains were removed immediately, a coronal brain slice (approximately 3 mm thick and 4 mm from the frontal pole) was cut with a blade, and the right basal ganglia was obtained; a portion of the tissue was fixed in 10% buffered formalin, and the remainder was immediately stored at -70 °C until analysis.

2.2. Animal model preparation

Due to the fact that intracerebral hemorrhage often occurs in the basal ganglia, a rat model of ICH in right basal ganglia was used (Wan et al., 2009; Nakamura et al., 2010). The rat was anesthetized with intraperitoneal sodium pentobarbital (50 mg/kg) and positioned in a stereotactic frame. The scalp was incised along the midline and the body (rectal) temperature was maintained at 37 °C during the surgery using a feedback-controlled heating system. Using a sterile technique, a 1 mm burr hole was performed in the skull on the right coronal suture 3 mm lateral to the midline. A blunt 26-gage needle was inserted into the right basal ganglia under stereotactic guidance (coordinates: 0.2 mm anterior, 6.0 mm ventral, and 3.0 mm lateral to the midline). Then, a 75 μL of autologous whole blood was infused at a rate of 20 μL/min via a microinfusion pump. After finishing the infusion, the needle was withdrawn quickly, cyanoacrylate glue was placed around the burr hole, and the skin incision was closed with sutures. For the rats in the sham operation group, only 75 μL normal saline was injected. After these procedures, the rat was returned to cage and room temperature was regulated to 23 ± 1 °C.

2.3. OMT preparation

OMT was purchased from Shanxi Huike Botanical Development Company Limited (Shanxi, China), with a purity of 98%. A stock suspension at 40 mg of OMT per ml was prepared with distilled water and stored at 4 °C.

2.4. Reverse transcription-polymerase chain reaction analysis

Total RNA was extracted with Trizol (Invitrogen) and its density and purity were detected. RNA (2 μg) of each sample was used for synthesizing cDNA through inverse transcription; 1 μL of cDNA was used to carry out PCR amplification (BioRad, Hercules, CA, USA). Primers were synthesized by Shanghai Sangon Biological Engineering Technology Company Limited. Correctness of the gene order was proved in GenBank. Primers included 5′-GCC GGA AAG TTA TTG TGG TGG T-3′ (forward) and 5′-ATG GGT TTT AGG CGC AGA GTT T- 3′ (reverse) for TLR4, 5′-GCG CAT CCA GAC CAA CAA TAA C-3′ (forward) and 5′-GCC GAA GCT GCA TGG ACA CT- 3′ (reverse) for NF-κB, as well as 5′-GCC ATG TAC GTA GCC ATC CA-3′ (forward) and 5′-GAA CCG CTC ATT GCC GAT AG -3′ (reverse) for β-actin. PCR conditions were initial denaturation for 2 min at 95 °C, 35 cycles of amplification with denaturation at 95 °C for 30 s, annealing at 52 °C for 30 s, and extension at 72 °C for 40 s. After 35 cycles, extension at 72 °C for 5 min was done. RT-PCR products (5 μL) were analyzed by 2% agarose gel electrophoresis. The gray scale of the electrophoresis strip was scanned by an ultraviolet photometry gel imaging system (Leica, Cambridge, England). The relative expression of products was represented with TLR4/β-actin and NF-κB/β-actin; data were analyzed with an image analysis system (Leica, Cambridge, England).

2.5. Determination of inflammatory cytokines in hemorrhagic brain tissue

The tissues were homogenized at a concentration of 40% weight/volume with 0.01 mol/L phosphate-buffered saline, pH 7.4, containing a protease inhibitor cocktail (Roche, Indianapolis, IN). The homogenates were then centrifuged at 7500 g for 20 min at 4 °C. The levels of TNF-α, IL-1β, and IL-6 in the supernatant were determined using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, Minn, USA) by an ELISA analyzer (Coda, Bio-Rad, USA). Results were expressed as picograms per milligram (pg/mg) of brain tissue.

2.6. TUNEL staining

The formalin-fixed brain tissues were embedded in paraffin and sectioned at 4 μm with a microtome. The sections were examined for apoptotic cells by the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) method, using an in situ cell death detection kit (ISCDD, Boehringer Mannheim, Germany). Briefly, sections were deparaffinized, rehydrated, and washed with distilled water. The tissue was digested with 20 μg/ml proteinase K (Boehringer Mannheim, Mannheim, Germany) at room temperature for 15 min. Endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide/methanol in PBS at 37 °C for 30 min. The sections were then incubated with terminal deoxynucleotidyl transferase at 37 °C for 60 min to add dioxigenin-conjugated dUTP to the 3′-OH ends of fragmented DNA. Anti-dioxigenin antibody peroxidase was applied to the sections in order to detect the labeled nucleotides. The sections were stained with DAB and counterstained lightly with hematoxylin. The apoptotic cells were identified and counted by light microscopy (OLYMPUS, Japan), performed by an investigator that was blinded to the treatments. The extent of neurological damage was evaluated by the apoptotic index (i.e., the mean number of positive neuronal cells per 100 neuronal cells, based on examination of 1000 neuronal cells).

2.7. Western blot analysis

Protein extract for 12/15-LOX, phospho-p38 MAPK, and cPLA2 was obtained using a total protein extraction kit (Applygen Technologies Inc., Beijing, China). Protein concentration of the supernatant was measured using a BCA Protein Assay Reagent Kit (Novagen, Madison, WI, USA). Equal amount of protein samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred on to nitrocellulose membranes (Millipore, Billerica, MA, USA). The membrane blots were blocked with 5% milk in TBS-T (0.1 M Tris-HCl, pH 7.4, 0.9% NaCl, 0.1% Tween-20) and incubated with primary antibodies against 12/15-LOX, phospho-p38 MAPK, and cPLA2 (Bioworld Technology, Minneapolis, MN, USA) at 4 °C overnight. Fluorescent-labeled secondary antibody (Rock-land, Gilbertsville, PA, USA) was used to incorporate primary antibodies. Relative density was analyzed by an imaging densitometer (LI-COR Bioscience, Lincoln, NE, USA). The densitometric values were normalized by β-actin immunoreactivity (Santa Cruz Biotech, Santa Cruz, CA), and used for statistical analysis.