Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.JournalofSurgicalResearch.com

Levobupivacaine inhibits lipopolysaccharide-induced high mobility group box 1 release *in vitro* and *in vivo*

Yunfen Ge, MD, Shuangfei Hu, MD, Yunlong Zhang, MD, Wenyuan Wang, PhD, Qiong Xu, MD, Leping Zhou, MD, and Hui Mao, MD*

Department of Anesthesiology, Zhejiang Provincial People's Hospital, Hangzhou, Zhejiang, China

ARTICLE INFO

Article history:
Received 8 April 2014
Received in revised form
28 May 2014
Accepted 29 May 2014
Available online 4 June 2014

Key Words:
levobupivacaine
HMGB1
Sepsis
Lipopolysaccharides
NF-κB
p38 MAPK

ABSTRACT

Background: The aim of the study was to investigate whether levobupivacaine (LB) suppressed lipopolysaccharide (LPS)-induced high mobility group box 1 (HMGB1) release *in vitro* and *in vivo*, and to determin its molecular mechanisms of action.

Materials and methods: RAW264.7 cells were treated with LPS and LB for 24 h. Levels of HMGB1, nuclear factor-kappa B (NF-κB) and phosphorylated p38 mitogen-activated protein kinase (MAPK) were measured by Enzyme-linked immunosorbent assay and Western blotting; the levels of HMGB1 messenger RNA were measured by real-time polymerase chain reaction. In addition, cecal ligation and puncture–induced septic C57BL/6 received LB infusion, and the levels of HMGB1 and functional parameters of multiple organs determined using several detection kits.

Results: LB inhibited HMGB1 release *in vitro* and *in vivo*. Furthermore, LB inhibited the translocation of NF-κB and phosphorylation of p38 MAPK *in vitro*. Mice treated with LB infusion improved survival in mice and significantly reduced cecal ligation and puncture –induced dysfunction of organs.

Conclusions: LB suppresses LPS-induced HMGB1 release *in vitro* and *in vivo* by partially inhibiting NF-κB/p38 MAPK pathways. LB can rescue mice from sepsis and protect against organ dysfunction in septic mice.

1. Introduction

Sepsis, a potentially life-threatening syndrome, is mediated by an early (e.g., tumor necrosis factor α [TNF-α] and interleukin 1β [IL-1β]) and late (e.g., high mobility group box 1 [HMGB1]) proinflammatory cytokine response to infection [1]. HMGB1 is a highly conserved nuclear nonhistone protein that exhibits diverse functions according to its cellular localization [2]. In the intracellular compartment, it participates in a number of biological functions such as gene transcription, DNA replication, and DNA repair [3]. In addition to its intracellular functions, extracellular HMGB1 plays a key part in inflammation when actively secreted from immune cells [4]. Recent evidence indicates that circulating HMGB1 levels are elevated in a delayed fashion in animal models of sepsis and in septic patients characterized by overwhelming inflammatory reactions, which mediate the systemic inflammatory response, multiple organ dysfunction, and lethality [5-7]. In fact, the administration of anti-HMGB1 antibodies or inhibitors, such as genipin and chlorogenic acid, has been

* *Corresponding author*. Department of Anesthesiology, Zhejiang Provincial People Hospital, No. 158 Shangtang Road, Hangzhou, Zhejiang, China. Tel.: +86 571 85239988; fax: +86 571 85131448.
E-mail address: shuangfeihu666@outlook.com (S. Hu).

0022-4804/$ – see front matter
http://dx.doi.org/10.1016/j.jss.2014.05.087

shown to protect mice against lipopolysaccharide (LPS)-induced acute tissue injury and lethal endotoxemia [8,9]. Therefore, anti-HMGB1 treatment may represent a therapeutic target for treating systemic inflammatory diseases.

Local anesthetics have been reported to possess anti-inflammatory properties [10,11]. Previous data have confirmed that bupivacaine, a widely used local anesthetic agent, inhibits a variety of inflammatory molecules, including cytokines, cyclooxygenase-2 and prostaglandin E_2 [10], by inhibiting activation of nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) signaling pathways [12]. Levobupivacaine (LB), the pure S (−)-enantiomer of racemic bupivacaine, has similar local anesthetic potency to bupivacaine; however, LB had a lower risk of cardiovascular and CNS toxicity than bupivacaine [13]. Recently, data indicated that LB could attenuate endotoxin-induced upregulation of inflammatory mediators in microglia [12]. However, few studies have assessed the effects of LB on HMGB1 expression and the functions of critical organs, such as the heart, liver, and kidney in septic mice. HMGB1 overexpression occurs in various inflammatory diseases, and LB has an inhibitory effect on NF-κB and MAPK signaling pathways, we hypothesized that LB might inhibit overexpression of HMGB1 during the inflammatory process. Therefore, we investigated whether LB inhibits the release and expression of HMGB1 from LPS-activated RAW264.7 cells and explored the mechanisms of these anti-inflammatory effects.

2. Materials and methods

2.1. Cell culture

The immortalized murine macrophage cell line RAW264.7 (Shanghai Institute of Cell Biology, The Chinese Academy of Sciences, Shanghai, China) was used to facilitate gene expression and HMGB1 production. Cell culture was performed according to previous studies [14-17]. In brief, RAW264.7 cells were cultured in Roswell Park Memorial Institute 1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT) and 1% of a ready-to-use and diluted penicillin–streptomycin solution (Life Technologies Corp, Carlsbad, CA) in a humidified cell incubator at 5% CO_2 and 37°C. When the cells reached 80%–90% confluence, they were washed three times with phosphate-buffered saline (PBS) and then transferred to six-well polystyrene culture plates at a density of 1×10^6 cells per well in 2 mL medium per well. After overnight incubation, the medium was removed and replaced with Roswell Park Memorial Institute 1640 medium containing 0.25% fetal bovine saline (for experiments designed to measure HMGB1 in conditioned media).

2.2. Experimental protocol

Two groups of confluent RAW264.7 cells allocated to receive to LPS (500 ng/mL; *Escherichia coli serotype* 0111:B4; Sigma–Aldrich, St. Louis, MO) or normal saline were used as positive or negative controls and were designated as LPS and C groups, respectively. To elucidate the effects of LB, three groups of confluent RAW264.7 cells were allocated to receive LPS, immediately followed by LB (5, 25, and 50 μM; Sigma–Aldrich) and were designated as LPS + LB (5), LPS + LB (25), and LPS + LB (50) groups, respectively. To control for the effects of LB, another group of confluent RAW264.7 cells was allocated to receive LB (50 μM) and was designated the LB group. After reacting with LPS for 24 h, cell cultures were harvested for subsequent analysis. The concentrations of drug were determined according to a previous study [12].

2.3. Enzyme-linked immunosorbent assay

The culture medium from each sample was collected and the concentrations of HMGB1 in each sample were then assayed using enzymed-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN) and were performed according to the manufacturer's instructions.

2.4. Cell viability

The viability of RAW264.7 cells was determined using a Cell Counting Kit-8 (CCK-8) Assay kit (Beyotime, Wuhan, China) as reported previously [17]. RAW264.7 cells (1×10^4 cells per well) were seeded in 96-well plates and incubated at 37°C for 24 h. Cells were treated with various concentrations of LB or vehicle (normal saline) and incubated at 37°C for an additional 24 h. After incubation, 20 μL CCK-8 reagents were added to each well, and the plates were further incubated for 3 h at 37°C. The optical density of each well was measured at 450 nm using a microplate reader (Bio-Rad Laboratories, Hercules, CA) at a test wavelength of 450 nm and a reference wavelength of 630 nm.

2.5. Cell death assay

RAW264.7 cells (1×10^4 cells per well) were incubated with or without LB treatment for 24 h and then lysed for use in the Cell Death Detection ELISA^{PLUS} Assay (Roche Applied Science, Penzberg, Germany) according to the manufacturer's instructions [16]. This kit is a photometric enzyme immunoassay for *in vitro* qualitative and quantitative determination of the cytoplasmic histone–associated DNA fragments (mononucleosomes and oligonucleosomes) generated by apoptotic cell death using mouse monoclonal anti-histone and anti-DNA antibodies. Positive and negative controls were included as provided by the manufacturer .Absorbance was measured using a microplate reader at a wavelength of 405 nm.

2.6. Real-time polymerase chain reaction analysis

Total RNA was extracted from RAW264.7 cells using TRIzol reagent (Invitrogen, Carlsbad, CA). Complementary DNA was synthesized from 1 μg of RNA using reverse transcriptase with oligo (dT) primer (Invitrogen). Real-time polymerase chain reaction (PCR) was performed using an SYBR Green PCR kit (Roche, Mannheim, Germany) on a 7300 real-time instrument (Applied Biosystems, Foster City, CA). The sequences of the primers were as follows: HMGB1 forward, CAC CGT GGG ACT ATT AGG AT; HMGB1 reverse, GCT CAC ACT TTT GGG GAT AC; β-actin forward, CCT CTA TGC CAA CAC AGT; β-actin reverse,