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The SMAC mimetic birinapant attenuates lipopolysaccharide-induced liver injury by inhibiting the tumor necrosis factor receptor–associated factor 3 degradation in Kupffer cells

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ABSTRACT

It was demonstrated that second mitochondria-derived activator of caspases (SMAC) mimetic inhibites tumor necrosis factor receptor-associated factor 3 (TRAF3) degradation and the mitogen-activated protein kinase (MAPK) signaling pathway activation induced by lipopolysaccharide (LPS) in vitro. However, the effect of Smac mimetic in vivo is not clear. The present study was to investigate the role of Smac mimetic in LPS-induced liver injury in mice and its possible mechanism. An animal model of LPS-induced liver injury was established by intraperitoneally injecting mice with 10 mg/kg LPS pretreatment with or without Smac mimetic birinapant (30 mg/kg body weight). Birinapant significantly improved the survival rate of endotoxemic mice ($P < 0.05$) and attenuated LPS-induced liver pathologic damage and inflammatory response. IL-1 and TNF-α levels in the serum were markedly decreased in birinapant pretreatment mice compared with control mice ($P < 0.05$).The cellular inhibitor of apoptosis protein 1 (cIAP1) expression in liver resident macrophage (Kupffer cells, KCs) was significantly decreased in the Birinapant group compared to the Vehicle group ($P < 0.05$). At the same time, total TRAF3 protein abundance in KCs rapidly declined after LPS stimulation in the Vehicle group. However, it remained constant in the Birinapant group. Moreover, K48-linked polyubiquitination of TRAF3 in KCs was markedly impressed in the birinapant group compared with the control group. At last, the JNK and p38 MAPK activation in KCs was significantly inhibited by birinapant pretreatment ($P < 0.05$). These results suggested that birinapant attenuated liver injury and improved survival rates in endotoxemic mice by inhibited the expression of cIAP1, degradation of TRAF3 and aviation of MAPK signaling pathway.

1. Introduction

Lipopolysaccharide (LPS) is a major component of the outer membrane of Gram-negative bacteria, which triggers the rise of inflammatory cytokines and reactive oxygen species and plays a key role in liver injury in sepsis [1,2]. LPS-induced liver injury is mainly attributed to inflammatory mediators produced by activated macrophages, including tumor necrosis factor-α (TNF-α)and interleukins-1(IL-1), IL-6, IL-8, and IL-12 [3]. As the largest population of inherent macrophages in liver, Kupffer cells (KCs) reside in hepatic sinusoid and constitute the firstline of defense against gut-derived bacteria, microbial debris, and bacterial endotoxins [4–6]. When activated by LPS, KCs release inflammatory cytokines and play a critical role in the pathogenesis of various liver diseases range from LPS induced liver injury [7] to liver ischemia reperfusion injury [8]. Toll-like receptor4 (TLR4) is a pattern-recognition receptor (PRR) that is expressed on the cell surface of KCs and responsible for the sensing of LPS [9]. LPS-induced TLR4 engagement leads to the recruitment of multprotein complexes, which include Myeloid differentiation primary response protein 88 (MyD 88), TNF receptor–associated factor 6 (TRAF6), TRAF3, and cellular inhibitors of apoptosis proteins(cIAPs) [10]. Complex formation results in TRAF6 activation and K63-linked polyubiquitination of cIAPs, which enhances the K48-specific ubiquitin ligase activity toward TRAF3. The K48-linked polyubiquitination

Abbreviations: KCs, Kupffer cells; SMAC, second mitochondria-derived activator of caspases; TRAF3, tumor necrosis factor receptor–associated factor 3; MAPK, mitogen-activated protein kinase (MAPK); LPS, lipopolysaccharide; TNFα, tumor necrosis factor alpha; IL-1, interleukin-1; cIAP1, cellular inhibitor of apoptosis protein 1; TLR4, Toll-like receptor4; PRR, pattern-recognition receptor; MyD88, Myeloid differentiation primary response protein 88.

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of TRAF3 results in protein degradation and cytosolic translocation of the MyD88 complex, which activates mitogen-activated protein kinases (MAPKs) signaling pathway and induces inflmmatory genes expression [11,12].

cIAPs is a family of proteins which possess baculoviral IAP repeats domains that mediate binding to post-mitochondrial caspases, including XIAP, cIAP1, cIAP2, ML-IAP, and survivin [13]. They are regulated by the second mitochondria-derived activator of caspases (SMAC) which is released from mitochondria upon onset of apoptosis and binds directly to cIAPs leading to their degradation [14]. Many cIAP inhibitors were designed to function as SMAC mimetics for cancer treatments. However, studies about the role of SMAC mimetics in inflammatory reactions are rare. Tseng et al. demonstrated that SMAC mimetic inhibites TRAF3 degradation and the MAPK signaling pathway activation induced by LPS in RAW264.7 cells. However, the effect of Smac mimetic on LPS associated inflammatory respones in vivo is yet to be known. Birinapant is a SMAC mimetic designed to specifically target cIAP1 and cIAP2 for degradation [15]. In the present study, we evaluated the effects of birinapant on LPS-induced liver injury and the possible mechanism.

2. Materials and methods

2.1. Reagents

The primary antibodies for TRAF3, cIAP1, p38, phospho (p)-p38, JNK, p-JNK and Actin were purchased from Abcam (Cambridge, MA, USA). The primary antibodies for K48 linked-ubiquitin was purchased from Millipore (Billerica, MA, USA). The mouse enzyme-linked immunosorbent assay (ELISA) kits for the detection of TNF-α, and IL-1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Ultrapure Escherichia coli 0111:B4 LPS was obtained from Sigma-Aldrich (St. Louis, MO, USA). Birinapant was purchased from Selleckchem (Burlington, ON, Canada).

2.2. Animals and groups

Male C57BL/6J mice aged 8 week old, weighing 21 ± 1.3g, were provided by Experimental Animal Center of Chongqing Medical University. All the animals were received humane care in accordance with the National Institutes of Health guidelines for animal research and the legal requirements in China. The liver-injury model was induced by intraperitoneal injection of LPS (10 mg/kg body weight). Before the LPS administration, mice were injected intraperitoneally with birinapant (30 mg/kg body weight, birinapant group) either vehicle control (vehicle group) for 24 h [15]. Birinapant was dissolved in 12.5% Captisol (Ligand Pharmaceuticals) in distilled water. Twenty-four mice in each group were euthanized and the samples of the liver and blood were harvested at 0, 6, 12 and 24 h after LPS challleage (n = 6/subgroup). Primary KCs were isolated from mouse liver using a three-step procedure as described previously [16]. Cells were cultured in 6-well plates at a density of 3–4 × 10^5 cells/well in Dulbecco's modified Eagle's medium (DMEM) (Hyclone, USA). Twelve mice in each group were set aside for survival observation up to 72 h after LPS injection (45 mg/kg body weight). Survival experiments were replicated three times independently.

2.3. Histopathological study

Liver tissues were fixed in 10% neutral formalin and cut into 5-μm-thick sections. Paraffin sections were stained with haematoxylin and eosin (HE). The sections for immunohistochemistry staining were stained using commercial kits (ZSGB-Bio, Beijing, China) following the manufacturers' instructions. The quantitative immunohistochemical staining values (QISV) were calculated as the integrated optical density divided by the total area occupied by the brown and blue cells in each slide.

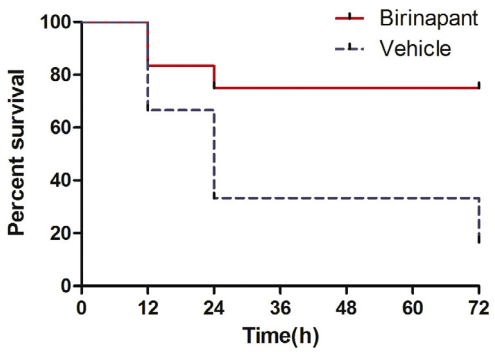

Fig. 1. Survival rates after LPS injection (45 mg/kg). Birinapant prevent LPS-induced death in mice (P < 0.05). Each group included 12 mice. Survival experiments were replicated three times independently.

2.4. Co-immunoprecipitation and immunoblotting

The K48-linked polyubiquitination of TRAF3 in KCs was determined by co-immunoprecipitation. The TRAF3 and cIAP1 protein expression, JNK and p38 MAPK activation in KCs were detected by immunoblotting. Complex co-immunoprecipitation was performed using protein G-agarose beads as described previously [17]. For protein ubiquitination analyse, total KCs lysates were prepared using an ice-cold lysis buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, 0.2% Nonidet P-40 and 20 mM N-ethylmaleimide (NEM, Sigma). For immunoblotting, total protein of the KCs was extracted by cell lysis buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0. 2 mM EDTA, 1 mM PMSF and 20 μg/ml aprotinin. Proteins for immunoblotting were separated using SDS-polyacrylamide gel electrophoresis in a Bio-Rad Mini protean apparatus (Bio-Rad, Hercules, CA, USA) and electrotransferred to PVDF membranes (Millipore, Billerica, MA, USA). Membranes were blocked and probed with anti-TRAF3, anti-cIAP1, anti-p38, anti-phospho (p)-p38, anti-JNK, anti-p-JNK and anti-Actin primary antibodies overnight at 4 °C and was incubated with secondary antibody for 2 h at room temperature. The relative amount of protein was quantified from relative optical density of the band by Image J software.

2.5. Enzyme-linked immunosorbent assay (ELISA)

ELISA was used to determine TNF-α and IL-1 level in the plasma according to the protocol.

2.6. Statistical analysis

All data were shown as mean ± standard deviation and analyzed with SPSS17.0 software. Student's *t*-test was used for single comparisons. P values < 0.05 were considered significant.

3. Results

3.1. Birinapant pretreatment significantly improved mice survival

A Kaplan–Meier model was constructed from the data to compare overall survival rates between the two groups. The mean survival time of Birinapant group (58.0 h) was significantly longer than that of Vehiche group (36.0 h), (P < 0.01, Fig. 1). Injection of LPS resulted in a 72 h survival rate of only 16.7%. In contrast, birinapant