Bilirubin alleviates alum–induced peritonitis through inactivation of NLRP3 inflammasome

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A R T I C L E   I N F O

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A B S T R A C T

Bilirubin is an endogenous substance derived from heme catabolism. In this study, we aimed to assess the anti-inflammatory activity of bilirubin, and to determine the mechanism thereof. The anti-inflammatory activity of bilirubin was evaluated using lipopolysaccharide (LPS)-treated peritoneal macrophages (PMs) and Raw264.7 cells, and mice with alum-induced peritonitis. The mRNA and proteins of NOD-like receptor family pyrin domain containing 3 (Nlrp3) and inflammatory cytokines were determined using qPCR and Western blotting, respectively. Distribution of phosphorylated (p) p65 (a NF-κB subunit) in the cytoplasm and nucleus were evaluated by immunofluorescence analysis and electrophoretic mobility shift assay. Bilirubin prior to LPS treatment decreased protein expressions of Nlrp3, pro-interleukin (IL)-1β and mature IL-1β in PMs, whereas bilirubin post LPS treatment showed no effects. Bilirubin prior to LPS treatment dose-dependently repressed expressions of Nlrp3 and IL-1β, and inhibited translocation of p-p65 to nucleus in Raw264.7 cells. Bilirubin treatment decreased myeloperoxidase activity and reduced the levels of inflammatory cytokines (i.e., IL-1β, TNFα and IL-6) in lavage fluid in mice with alum-induced peritonitis. This was accompanied by a lower mortality rate. In addition, the mRNAs of Nlrp3 and IL-1β in peritoneal exudates cells were decreased, and the levels of p-p65 and mature IL-1β proteins were reduced. In conclusion, bilirubin acted on inflammation and alleviated alum–induced peritonitis through inactivation of Nlrp3 inflammasome.

1. Introduction

Bilirubin is a chemical structure produced by heme catabolism in mammals, and its concentration ranges from 0.2 to 1.2 mg/dl in human serum [1]. Bilirubin is highly neurotoxic when at high levels [2]. Multiple steps are involved in bilirubin detoxification that controls bilirubin to a low safe range. The produced bilirubin in blood is transported into hepatocytes via OATP1B1 and OATP1B3. In the hepatocytes, bilirubin is metabolized to the glucuronide forms (i.e., conjugated bilirubin) by UGT1A1. The conjugated bilirubin is excreted to bile (biliary clearance) mainly via MRP2 and to blood circulation (for renal clearance) via MRP3 [3]. Genetic deficiency of UGT1A1 or MRP2 (the lack of bilirubin detoxification ability) is associated with various forms of hyperbilirubinemia such as Crigler-Najjar, Gilbert and Dubin–Johnson syndromes [4,5].

Although being toxic at a high level, bilirubin at a low level may possess health benefits [6–8]. For instance, bilirubin can decrease the level of adiposity in the livers of obese people, and shows a cell protection effect through inhibition of reactive oxygen species in lipid metabolism [6]. Moreover, previous studies suggest an anti-inflammatory activity for bilirubin [7,8]. Although bilirubin plays a potential role in inflammation responses, the mechanism of action remains elusive.

NOD-like receptor (NLR) family pyrin domain containing 3 (NLRP3, a member of the NLR family of cytosolic pattern recognition receptors) inflammasomes plays a crucial role in innate immune responses to pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) [9]. NLRP3 inflammasome is a large complex containing various pattern recognition receptors and adaptor molecules that play an essential role in the development of inflammatory responses and the clearance of pathogens in the innate immune system [9].

Abbreviations: LPS, lipopolysaccharide; NLRP-3, NOD-like receptor family pyrin domain containing 3; NF-kB, nuclear factor-kB; EMSA, electrophoretic mobility shift assay; MPO, myeloperoxidase; qPCR, real-time polymerase chain reaction; PM, peritoneal macrophages; IL, interleukin; MRP, multidrug resistance-associated protein; UGT, UDP-glucuronosyltransferase; OATP, organic-anion-transporting polypeptide; PAMPs, pathogen-associated molecular patterns; DAMPs, danger-associated molecular patterns; ASC, apoptosis-associated speck-like protein containing a CARD; TNF-α, tumor necrosis factor alpha; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; PBS, phosphate buffer solution

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protein complex consisting of NLRP3, apoptosis-associated speck-like protein containing a CARD (ASC) and caspase-1 [10]. Activation of NLRP3 inflammasome involves two sequential steps (i.e., priming and assembling) trigged by two signals [9]. The priming step triggered by the first signal (e.g., a PAMP such as lipopolysaccharide (LPS)) activates nuclear factor-κB (NF-κB) to induce the transcription of pro interleukin (IL)-1β and NLRP3. The second signal (e.g., a DAMP) triggers several signaling pathways that induce the assembly of NLRP3 inflammasome using its three components [10]. Activation of NLRP3 inflammasome promotes the cleavage of caspase-1 and maturation and secretion of pro-inflammatory cytokines IL-1β and IL-18 [11].

As noted above, NF-κB plays an important role in priming of NLRP3 inflammasome. NF-κB knockout in mice leads to deficiency in immune responses such as impaired B cell proliferation [12]. NF-κB signaling requires NF-κB and Rel family proteins. NF-κB family proteins include NF-κB1 (p50 and p105) and NF-κB2 (p52 and p100), whereas Rel proteins include RelA (p65), RelB and C-Rel. NF-κB and Rel proteins form dimers (e.g., p50/p65 dimer) that undergo phosphorylation and enter the nucleus to regulate the transcription of target genes such as NLRP3 and IL-1β [9,13].

In this study, we aimed to assess the anti-inflammation activity of bilirubin, and to determine the mechanism thereof. The anti-inflammation activities of bilirubin were evaluated using LPS-treated peritoneal macrophages and Raw264.7 cells, and mice with alum-induced peritonitis. The mRNA and proteins of Nlrp3 and inflammatory cytokines were determined using qPCR and Western blotting, respectively. Distribution of phosphorylated p65 in the cytoplasm and nucleus were evaluated by immunofluorescence analysis and electrophoretic mobility shift assay (EMSA). Our study for the first time demonstrated that bilirubin acted on inflammation and alleviated alum-induced peritonitis through inactivation of Nlrp3 inflammasome.

2. Materials and methods

2.1. Materials

Murine Raw264.7 cells were purchased from American Type Culture Collection (Manassas, VA). The ELISA assay kits for IL (interleukin)-6, IL-1β and Tnfα (tumor necrosis factor α) were purchased from Meimian Biotechnology (Yancheng, Jiangsu, China). The biochemical assay kit for myeloperoxidase (MPO) was obtained from Jiancheng Bioengineering Institute (Nanjing, China). BCA assay kit, biotin-labeled NF-κB probe, cytoplasmic/nuclear protein extraction kit, Bay11-7082, and EMSA kit were purchased from Beyotime (Shanghai, China). JetPrime transfection kit was purchased from POLYPLUS Transfection (Illkirch, France). Hybond-N + membranes were purchased from Amersham (Buckinghamshire, UK). RNAiso Plus reagent was obtained from Takara (Tokyo, Japan). PrimeScript RT Master Mix was purchased from Vazyme (Nanjing, China). Bilirubin was obtained from Aladdin Chemicals (Shanghai, China). Anti-Gapdh was obtained from HuaBio (Hangzhou, China). The primary antibodies of phosphorylated (p)-p65, p65, p50, Caspase1 p20, IκBα, β-actin, Histone H3 and NLRP3 were purchased from proteintech (Wuhan, China). LPS (lipopolysaccharide) was purchased from Sigma-Aldrich (St. Louis, MO). Imject alum was purchased from Thermo Fisher Scientific (Waltham, MA).

2.2. Animal studies

C57BL/6 mice were obtained from Beijing HFK Bioscience (Beijing, China). All mice were bred and maintained on a 12 h light/ dark cycle, with free access to food and water at the Institute of Laboratory Animal Science (Jinan University, Guangzhou, China). The mice (7–8 week old, male, n = 8) were randomly divided into two groups, namely, treatment and control groups. The treatment and control groups of mice respectively received intraperitoneal injections of bilirubin (30 mg/kg, b.i.d) and vehicle for three consecutive days. After last dosing on day 3, all mice were administered with alum (i.p., 700 μg). After 12 h, the peritoneal cavities of mice were washed with 6 ml of sterilized PBS (phosphate-buffered saline). The lavage fluid (containing peritoneal exudates cells (PECs)) was collected, followed by ELISA analyses (IL-6, IL-1β, TNF-α and MPO), qPCR and Western blotting.

2.3. Cell culture and treatment

Raw264.7 cells were cultured in DMEM containing 10% FBS, and used in two types of experiments. In first set of experiments, the cells were seeded into 12-well plates. Once reaching a confluence of 80–90%, cells were pre-treated with bilirubin (5 or 10 μM) or vehicle for 1 h, followed by LPS treatment (500 ng/ml). After 8 h, the cells were collected for qPCR and Western blotting. In the second set of experiments, the cells were pre-treated with bilirubin (2.5, 5 and 10 μM) or Bay11-7082 or vehicle for 1 h, followed by LPS treatment (500 ng/ml). After 8 h, the cells were collected for EMSA assays.

2.4. Isolation of peritoneal macrophages

Peritoneal macrophages (PMs) were isolated from mice as previously described [9]. In brief, mice received an intraperitoneal injection of 4% thioglycollate broth. On day 5, mice were administered with 1640 medium containing 10% FBS (i.p., 10 ml). 30 min later, mice were sacrificed for collection of peritoneal fluid. PMs were seeded into 12-well plates. In the first set of bilirubin treatment experiments (before LPS treatment), PMs were treated with bilirubin (10 μM) or vehicle for 3 h, followed by the addition of LPS (a final concentration of 500 ng/ml) and allowed for 12 h incubation. Then, ATP (a final concentration of 2 mM) was added to the incubation medium. 0.5 h later, the cells were collected and processed for Western blotting. In the second set of experiments (after LPS treatment), PMs were treated with LPS (a final concentration of 500 ng/ml) for 3 h, followed by incubation with 1640 medium containing bilirubin (10 μM) or vehicle for 12 h. Then, ATP (a final concentration of 2 mM) was added to the incubation medium. 0.5 h later, the cells were collected and processed for Western blotting.

2.5. qPCR assay

The procedure was previously described [9]. RNA was extracted from cells using RNAiso Plus regents (Takara, Tokyo, Japan). Reverse transcription reactions were performed using PrimeScript RT Master Mix (Vazyme, Nanjing, China). The cDNA products were amplified using SYBR Green PCR Master Mix (Vazyme, Nanjing, China). The amplification program consisted of an initial denaturation at 95 °C for 5 min, 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. Rpib was used as an internal control. The 2−ΔΔCT method was used to calculate relative gene expression. The sequences of qPCR primers were listed in Table 1.
2.6. Immuno-fluorescence analysis

Immuno-fluorescence assay was performed as previously described [9]. Raw264.7 cells were permeabilized with acetone for 1 min and blocked with 10% normal swine serum for 10 min. The cells were then incubated with NF-κB phosphor (p)-p65 primary antibody overnight, and probed with Alexa Fluor 488-conjugated anti-rabbit antibody. After 1 h, 1 ml 4′,6-diamidino-2-phenylindole (Biotium, Freemont, CA) was added to the culture flask. 20 min later, cells were washed with PBS. Fluorescent images were obtained using a laser scanning microscope (Carl Zeiss, Oberkochen, Germany).

2.7. EMSA assay

The EMSA experiment was performed using an EMSA kit as previously described (Beyotime, Shanghai, China) [9]. In brief, nuclear proteins were extracted from Raw264.7 cells using a cytoplasmic/nuclear protein extraction kit (Beyotime, Shanghai, China), and were incubated with biotin-labeled NF-κB probe for 20 min. Then, the protein-DNA complex were loaded to 4% nondenaturing polyacrylamide gels for electrophoresis, followed by transfer onto the Hybond-N+ membranes. The bands were visualized using Omega Lum G imaging system (Aplegen, San Francisco, CA) and enhanced chemiluminescence (Vazyme, Nanjing, China).

2.8. Western blotting

The assays were performed as previously described [9]. The protein samples were loaded onto 10% sodium dodecyl sulfate polyacrylamide gel and subjected to electrophoresis. The products were transferred onto the polyvinylidene difluoride membranes. After blocking with 5% skim milk, the membranes were incubated with primary antibodies overnight, followed by incubation with horseradish peroxidase conjugated secondary antibody for 1 h. The bands were visualized using Omega Lum G imaging system (Aplegen, San Francisco, CA) and enhanced chemiluminescence (Vazyme, Nanjing, China).

2.9. Cell viability assay

The cytotoxicity experiments of bilirubin (10 μM) in mouse peritoneal macrophage and Raw264.7 cells were performed using MTT assays. Cells were seeded in 96-well plates and maintained in the 1640 RPMI or DMEM medium (containing 10% FBS). After 24 h, the medium was changed to fresh medium (containing 10 μM bilirubin) for 48 h. 10 μl MTT solution was added to the medium of each well. Then the cells were maintained in the dark. After 4 h, the MTT medium of each

Table 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5′-3′ sequence)</th>
<th>Reverse (5′-3′ sequence)</th>
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<tr>
<td>Nlrp3</td>
<td>ATTACCCGCCCGAGAAGG</td>
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<tr>
<td>IL-1β</td>
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</tr>
<tr>
<td>Ppib</td>
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<td>CAAAAGGAAGAGCAGGGACG</td>
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Fig. 2. Bilirubin inactivates Nlrp3 inflammasome in PMs (A) The viability of PMs after treatment of 10 μM bilirubin or vehicle. (B) The protein expressions of Nlrp3 in PMs treated with vehicle or LPS. (C) The protein expressions of Nlrp3, pro-IL-1β, IL-1β and Caspase-1(p20) in PMs treated with bilirubin (10 μM) or vehicle prior to or after LPS priming (500 ng/ml) (left panel). Quantification of Nlrp3, Pro-IL-1β and IL-1β protein expressions in PMs. Data are mean ± SD (n = 5 mice) (right panel). (D) mRNA measurements of Nlrp3 and inflammatory factors in PMs treated with different concentrations of bilirubin or vehicle prior to LPS priming (500 ng/ml) by qPCR. *P < 0.05.
well was changed to 100 μl DMSO and cells were incubated for 15 min at room temperature. The OD values were detected at the wavelength of 570 nm. Cell viability at 10 μM bilirubin was normalized to that at DMSO (as a control group).

Fig. 3. Bilirubin suppresses Nlrp3 inflammasome priming in Raw264.7 cells. (A) The viability of Raw264.7 cells after treatment with 5 and 10 μM bilirubin or vehicle. (B) mRNA measurements of Nlrp3 and inflammatory factors in Raw264.7 cells treated with different concentrations of bilirubin or vehicle prior to LPS priming (500 ng/ml) by qPCR. (C) Nlrp3 and pro-IL-1β protein expressions in Raw264.7 cells treated with bilirubin (5 or 10 μM) or vehicle prior to LPS priming (500 ng/ml) (left panel). Right panel shows quantification data of NLRP3 and Pro-IL-1β protein expressions. Data are mean ± SD (n = 5). *P < 0.05. (D) mRNA measurements of Nlrp3 and inflammatory factors in Raw264.7 cells treated with different concentrations of bilirubin or vehicle prior to LPS priming (500 ng/ml) by qPCR.

Fig. 4. Bilirubin suppresses the expression of p-p65. (A) The expression of p-p65 and IκBα in Raw264.7 cells treated with bilirubin (5 or 10 μM) or vehicle prior to LPS priming (500 ng/ml). (B) Quantification of p-p65 and IκBα protein expression in Raw264.7 cells treated with bilirubin (5 or 10 μM) or vehicle prior to LPS priming (500 ng/ml). Data are mean ± SD (n = 5). *P < 0.05.

2.10. Statistical analyses

Data are presented as mean ± SD (standard deviation). Statistical analyses were performed using Student’s t-test unless otherwise specified. The level of significance was set at p < 0.05 (*).
3. Results

3.1. Bilirubin inactivates Nlrp3 inflammasome in PMs

We first confirmed that bilirubin at a concentration of 10 μM is nontoxic to PMs (Fig. 2A). Bilirubin (10 μM) prior to LPS treatment decreased protein expressions of Nlrp3, pro-IL-1β and mature IL-1β in PMs, whereas bilirubin post LPS treatment showed no effects (Fig. 2B/C). The data suggested that bilirubin inhibited Nlrp3 inflammasome activity by acting on the priming stage and decreasing Nlrp3 expression (Fig. 2B/C). Moreover, Bilirubin exhibited concentration-dependent inhibition effects on inflammatory factor expressions in PMs (Fig. 2D).

3.2. Bilirubin suppresses Nlrp3 inflammasome priming in Raw264.7 cells

We confirmed that bilirubin at the concentrations of 5 and 10 μM is non-toxic to Raw264.7 cells (Fig. 3A). Bilirubin (5 and 10 μM) prior to LPS treatment dose-dependently repressed Nlrp3 and IL-1β mRNAs in Raw264.7 cells (Fig. 3B). Consistently, the protein levels of Nlrp3, pro-IL-1β were decreased (Fig. 3C). The data supported suppression of Nlrp3 inflammasome priming by bilirubin. Bilirubin also exhibited concentration-dependent inhibition effects on inflammatory factor expressions in Raw264.7 cells (Fig. 3D).

3.3. Bilirubin suppresses NF-κB signaling pathway

Bilirubin treatment before LPS stimulation dose-dependently reduced the expression of Ikbα and phosphorylated p65 in Raw264.7 cells (Fig. 4). LPS stimulation induced nuclear translocation of p65, p50 and phosphorylated p65 (p-p65) in Raw264.7 cells, as evidenced by Western blotting and immunofluorescence (Fig. 5). Bilirubin inhibited the translocation of p50, p65 and p-p65 to nucleus in the LPS-treated cells (Fig. 5). The effects of bilirubin on NF-κB signaling in Raw264.7 cells were further confirmed using EMSA assays (measuring the complex formation of NF-κB with a specific DNA probe). Bilirubin dose-dependently reduced formation of NF-κB–DNA complex (Fig. 6). As a positive control, the known NF-κB inhibitor Bay11-7082 markedly suppressed NF-κB–DNA complex formation (Fig. 6).
3.4. Bilirubin alleviates alun-induced peritonitis through inactivation of Nlrp3 inflammasome

The effects of bilirubin on inflammation were assessed using an alun-induced peritonitis model. Bilirubin alleviated alun-induced peritonitis as evidenced by decreased MPO activity and reduced levels of inflammatory cytokines (i.e., IL-1β, TNFα and IL-6) in lavage fluid (Fig. 7A/B). Alleviation of peritonitis was associated with a decreased mortality rate of mice (Fig. 7C). Furthermore, bilirubin decreased the mRNA levels of Nlrp3 and IL-1β in mouse PECs, suggesting down-regulation of NF-κB signaling pathway and potentially inactivation of Nlrp3 inflammasome (Fig. 8A). This was supported by the fact that p-p65, Nlrp3 and pro-IL-1β proteins were markedly reduced in PECs measured by Western blotting (Fig. 8B/C). Moreover, the IL-1β concentration was also significantly decreased by bilirubin in PMs incubation medium (Fig. 8D). Further, bilirubin reduced the protein expression of IL-1β p17 (mature IL-1β) in mice with peritonitis, indicating actual inactivation of Nlrp3 inflammasome (Fig. 8B/C). Collectively, our data supported that bilirubin alleviated alun-induced peritonitis through inactivation of Nlrp3 inflammasome.

4. Discussion

In this study, we observed anti-inflammatory effects of bilirubin in PMs and Raw264.7 cells (Figs. 2 & 3). The anti-inflammatory action of bilirubin was validated using alun-induced peritonitis model (Figs. 7 & 8). Further, we revealed that bilirubin acted on inflammation through regulation of Nlrp3 inflammasome activity. The evidence for regulation of Nlrp3 inflammasome by bilirubin was strong. First, bilirubin downregulated the expression of Nlrp3 and the production of mature IL-1β in LPS-treated PMs. Second, bilirubin decreased the expression of and nuclear translocation of phosphorylated p65 (a key factor in Nlrp3 inflammasome priming) in Raw264.7 cells. Third, bilirubin treatment caused reduced Nlrp3 and mature IL-1β in mice with alun-induced peritonitis (Fig. 8).

Previous studies and current one supported bilirubin as an anti-inflammatory agent [14]. In fact, bilirubin was also shown to possess other health benefits such as anti-oxidation and lipid-lowing effects [15]. However, bilirubin at a high concentration leads to severe neurotoxicity and even deaths [16]. Therefore, the beneficial versus detrimental effects of bilirubin may be highly dependent on the types of tissues and on the concentrations. Controlling bilirubin to a low level in the brain is necessary, whereas the levels of bilirubin in other tissues may be elevated to maximize its beneficial effects.

We demonstrated that bilirubin inactivated Nlrp3 inflammasome to reduce the severity of inflammation in the present study. However, a previous study showed that bilirubin inhibited the TNFα-related induction of three endothelial adhesion molecules (E-selectin, vascular cell adhesion molecule 1 and intercellular adhesion molecule 1) [17]. Zucker et al reported that bilirubin prevented acute DSS-induced colitis by inhibiting leukocyte infiltration and suppressing up-regulation of inducible nitric oxide synthase [18]. Therefore, the mechanisms of anti-inflammatory action for bilirubin appear to be multifaceted, involving inactivation of Nlrp3 inflammasome, down-regulation of TNFα pathway, and inhibition of leukocyte infiltration.

We proposed that bilirubin inactivated Nlrp3 inflammasome via acting on the priming stage rather than the assembly stage. This was because (1) bilirubin treatment prior to LPS priming decreased Nlrp3 and IL-1β expressions in PMs, whereas bilirubin treatment after LPS priming showed no effects (Fig. 2C); (2) bilirubin was identified as a negative regulator of NF-κB signaling that plays a key role in the priming of Nlrp3 inflammasome in Raw264.7 cells (Figs. 5 & 6); and (3) bilirubin treatment did not alter the expressions of ASC and caspase-1, two other components of NLRP3 inflammasome (data not shown).

Since bilirubin is a known neurotoxic agent, it was necessary to determine whether the compound is toxic to PMs and Raw264.7 cells.
(used for evaluations of the anti-inflammatory activity herein). Cell viability assays confirmed that bilirubin was non-toxic to PMs and Raw264.7 cells (Figs. 2A & 3A). Thus, it can be excluded that reduced levels of inflammatory factors by bilirubin was due to cytotoxicity. It was noteworthy that current study focused on the effects of bilirubin on Nlrp3 inflammasome activity. We acknowledged that whether bilirubin affected the activities of other inflammasomes were unknown.

The finding that bilirubin alleviated alum-induced peritonitis lent a strong support to the notion that bilirubin at low concentrations contributes to alleviations of certain inflammatory diseases such as colitis or endotoxin-induced pulmonitis [18,19]. However, alum-induced peritonitis is a typical NLRP3 inflammasome-dependent inflammation model [20,21]. Although we showed bilirubin alleviated alum-induced peritonitis, it is not necessary that bilirubin shows beneficial effects in the treatment of other types of peritonitis (e.g., zymosan-induced mouse peritonitis) that are independent of NLRP3 inflammasome [22].

In summary, bilirubin shows anti-inflammatory effects in PMs and Raw264.7 cells, as well as in mice with alum-induced peritonitis. The anti-inflammatory action of bilirubin was attained through down-regulation of Nlrp3 inflammasome activity.

**Conflict of interest**

The authors have declared that no conflict of interest exists.

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