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Dipyrithione inhibits lipopolysaccharide-induced iNOS and COX-2 up-regulation in macrophages and protects against endotoxic shock in mice

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Abstract Dipyrithione (PTS2) possesses anti-bacterial and anti-fungal activity. In the present study, we found that PTS2 dose-dependently inhibited the LPS-induced up-regulation of nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) protein level in RAW264.7 cells. RT-PCR experiments showed that PTS2 suppressed LPS-induced iNOS but not COX-2 expression at the mRNA level. As expected, PTS2 prevented NO secretion in RAW264.7 cells. Furthermore, PTS2 administration significantly decreased LPS-induced mortality in mice. Mechanistically, PTS2 decreased expression and phosphorylation of STAT1, but did not interfere with the MAPK and NF-κB pathways. In conclusion, PTS2 protects mice against endotoxic shock and inhibits LPS-induced production of pro-inflammatory mediators, suggesting that PTS2 could play an anti-inflammatory role in response to LPS.

Keywords: Dipyrithione; Lipopolysaccharide; iNOS; COX-2; STAT1; Endotoxic shock

1. Introduction

Lipopolysaccharide (LPS), a constituent of the gram-negative bacterial cell wall, induces inflammatory responses when administered to cells or animals, which is similar to those seen in septic shock, a serious circulatory disorder with a high mortality rate of 30–90% [1], indicating an apparent inefficiency of its current treatment [2,3]. Pro-inflammatory cells, mainly activated macrophages, are responsible for most of the cellular and molecular pathophysiology of sepsis by producing cytokines and other pro-inflammatory molecules including platelet-activating factor, prostaglandins, enzymes, and free radicals, such as nitric oxide (NO) [4–6]. Among a variety of inflammatory mediators, two of the most prominent are nitric oxide (NO) produced by inducible NO synthase (iNOS) and prostaglandins by cyclooxygenase-2 (COX-2; prostaglandin H2 synthase) [7,8]. NO may regulate almost all stages in the development of inflammation, in particular, the early stages of inflammatory cell transmigration to the sites of inflammation [9]. Prostaglandin E2 (PGE2), the production of COX-2, works as a common final mediator of the febrile [10]. Previous reports showed that when iNOS was up-regulated in inflammatory cells, COX-2 expression increased in a similar pattern [11], suggesting the interaction between iNOS and COX-2. Recently, it is reported that NO up-regulates COX-2 expression and iNOS binds, *s*-nitrosylates and activates COX-2 [12,13].

In various cells including macrophages, LPS stimulates toll-like receptor 4 (TLR4) to activate nuclear factor κB (NF-κB) that is an important transcription factor for iNOS and COX-2 [14]. LPS also had been shown to activate mitogen-activated protein kinases (MAPKs) pathways to enhance iNOS and COX-2 gene expression in macrophages [15–17]. Another transcription factor, the signal transducer and activator of transcription 1 (STAT1), is involved in LPS-induced iNOS expression, which is activated through the Janus kinase (JAK)–STAT-pathway [18,19].

Dipyrithione (2,2′-dithiobispyridine-1,1′-dioxide, PTS2) (CAS number: 3696-28-4), a pyrithione derivate (Fig. 1), is usually used as anti-bacterial and anti-fungal drug. Pyrithione (PTO), the monomer of PTS2, which inhibits the growth of fungi, yeast, mold, and bacteria, is widely used in cosmetics and shampoo. Recently, we reported that PTS2 induced HeLa cells apoptosis through activating MAPKs pathway [20]. Here, we show the evidence that PTS2 inhibits LPS-induced up-regulation of iNOS and COX-2 protein levels in RAW264.7 cells and protects mice against endotoxic shock. We also found that LPS-induced increase of iNOS but not COX-2 mRNA level was suppressed significantly by PTS2 treatment. This difference between the regulation effects of PTS2 on iNOS and COX-2 is related with the mechanism that PTS2 prevents

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Abbreviations: PTS2, dipyrithione; LPS, lipopolysaccharide; iNOS, inducible nitric oxide synthase; NO, nitric oxide; COX-2, cyclooxygenase-2; STAT1, signal transducers and activators of transcription 1; MAPKs, mitogen-activated protein kinases; NF-κB, nuclear factor κB; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase

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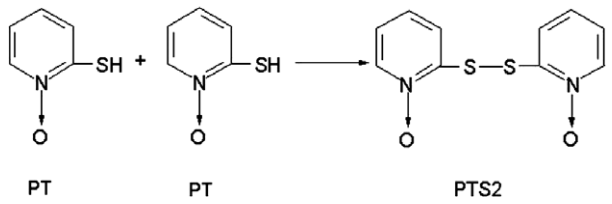

Fig. 1. Chemical structure of PTS2.

STAT1 phosphorylation in RAW264.7 cells. These results strongly suggest that PTS2 might exert anti-inflammatory activities in LPS-induced inflammation.

2. Materials and methods

2.1. Antibodies and reagents

Polyclonal antibodies against JNK/SAPK, phospho-JNK/SAPK (p-JNK), p38 MAPK, phospho-p38 MAPK (p-p38), ERK, phospho-ERK (p-ERK), IκB-α, phospho-IκB-α (p-IκB-α) and phospho-STAT1 (p-STAT1 Y701) were obtained from Cell Signaling Technology. Antibody to STAT1 and COX-2 were from Santa Cruz Biotechnology. The iNOS monoclonal antibody was purchased from BD Pharmingen. All secondary antibodies used for Western blotting were purchased from Calbiochem. LPS (from *Escherichia coli* 0111: B4) was purchased from Sigma. PTS2 was purchased from J&K Chemical Ltd.

2.2. Cell culture

RAW264.7, a murine macrophage-like cell line purchased from the CBCAS (Cell Bank of the Chinese Academic of Sciences, Shanghai, China) was maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% (v/v) fetal bovine serum (Hyclone) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) (HyClone) at 37 °C in an atmosphere of 5% CO_2.

2.3. Western blotting

Cells were rinsed twice with ice-cold PBS, and solubilized in lysis buffer containing 20 mM Tris (pH 7.5), 135 mM NaCl, 2 mM EDTA, 2 mM DTT, 25 mM β-glycerophosphate, 2 mM sodium pyrophosphate, 10% glycerol, 1% Triton X-100, 1 mM sodium orthovanadate, 10 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride (PMSF) for 30 min. Lysates were centrifuged (15000 $\times g$) at 4 °C for 15 min. Equal amounts of the soluble protein were denatured in SDS, electrophoresed on a 12% SDS–polyacrylamide gel, and transferred to a PVDF membrane. The immunoblotting were performed as described [21]. The horseradish peroxidase (HRP)-conjugated goat anti-mouse or anti-rabbit IgG antibodies were used against respective primary antibody. The proteins were visualized using Lumi-Light Western Blotting Substrate (Roche Molecular Biochemicals).

2.4. Reverse transcriptase-PCR (RT-PCR)

Total RNA was extracted with Trizol reagent (Gibco) as described by the manufacturer. RT-PCR was performed by Access RT-PCR System kit (Promega) according to the protocol with indicated primers (iNOS: sense primer 5′-cccttccgaagtttctggcagc-3′, antisense primer 5′-ggctgtcagagcctcgtggctt-3′; COX-2: sense primer 5′-tctccaacctctcctactac-3′, antisense primer 5′-gcacgtagtcttcgatcact-3′; GAPDH: sense primer 5′-tgaaggtcggtgtgaacggatttggc-3′, antisense primer 5′-tggttcacacccatcacaaacatgg-3′). PCR was performed for 30 cycles in 25 μl of reaction mixture. PCR products were visualized in 1.2 % agarose gels stained with EtBr. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was utilized as a housekeeping gene where indicated.

2.5. Nitrite analysis

NO synthesis was spectrophotometrically determined by assaying the culture supernatants for nitrite using the Griess reagent (1% sulfanilic acid, 0.1% *N*-1-naphthyl-ethylenediamine dihydrochloride, and 5% phosphoric acid). Absorbance was measured at 550 nm and nitrite concentration was determined using sodium nitrite as a standard.

2.6. Endotoxin shock model of mice

Male ICR mice weighing 18–22 g were purchased from Shanghai Experimental Animal Center, China Academy of Science. Laboratory animal handling and experimental procedures were performed in accordance with the requirements of Provisions and General Recommendation of Chinese Experimental Animals Administration Legislation and were approved by Science and Technology Department of Jiangsu Province. Each mouse was i.p. injected with LPS (37.5 mg/kg) in saline. Thirty minutes after LPS challenge the mice were administrated (i.p) with saline, PTS2 (1, 2.5 and 5.0 mg/kg) or dexamethasone (2 mg/kg), respectively. The survival rates were monitored continuously for 4 days.

2.7. Statistics

Analysis of variance (ANOVA) was used to compare the results between two groups. Individual points were compared using a Student's *t*-test and differences were considered significant for $P < 0.05$. Data are presented as means ± S.D. Western blotting analysis experiments were repeated 2–3 times with similar trends.

3. Results

3.1. PTS2 inhibits LPS-induced up-regulation of iNOS and COX-2 in RAW264.7 cells

iNOS and COX-2 are two of the inflammatory factors which are correlated with LPS stimulation. To investigate the anti-inflammatory activity of PTS2, we tested the effects of PTS2 on LPS-induced iNOS and COX-2 protein up-regulation in RAW264.7 cells by Western blotting. The cells were incubated for 8.5 h with 1.0–5.0 μM PTS2 30 min after LPS (100 ng/ml) pretreatment. The results showed that LPS-induced cellular iNOS and COX-2 protein increased dramatically and PTS2 inhibited the elevation of iNOS and COX-2 level in a dose-dependent manner. PTS2 alone even at concentration of 5.0 μM did not influence iNOS and COX-2 protein level in normal RAW264.7 cells (Fig. 2A). Aspirin is an aceylated salicylate used to treat inflammation and arthritis pain and has been reported to modulate LPS-induced NO release [22]. As shown in Fig. 2B, though 3 μM of both PTS2 and Aspirin inhibited LPS-induced iNOS and COX-2 expression effectively, PTS2 exhibited stronger inhibitory ability than Aspirin. In comparing PTS2 with its monomer, PTO, we found that PTS2 also acted more effective in inhibiting LPS-induced iNOS and COX-2 expression than PTO (Fig. 2C).

3.2. The effects of PTS2 on LPS-induced increase of iNOS and COX-2 mRNA level

As above results indicated that PTS2 inhibited the increase of iNOS and COX-2 protein level induced by LPS, we then performed RT-PCR to analyze the effects of PTS2 on LPS-induced iNOS and COX-2 expression at mRNA level. RAW264.7 cells were pretreated with LPS (100 ng/ml) for 30 min or not, and then incubated with 3.0 μM PTS2 for 8.5 h. Total RNA were isolated, iNOS and COX-2 mRNA was determined by RT-PCR. As shown in Fig. 3, LPS stimulation elevated endogenous mRNA level of iNOS and COX-2, whereas PTS2 suppressed LPS-induced increase of iNOS but not COX-2 mRNA level. These data suggested that PTS2 affected iNOS and COX-2 protein level through different route.