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-\(\kappa\)B 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.

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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, \(\text{s-nitrosylates and activates}\) COX-2 [12,13].

In various cells including macrophages, LPS stimulates toll-like receptor 4 (TLR4) to activate nuclear factor \(\kappa\)B (NF-\(\kappa\)B) 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
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'-tctccaacctctcctactac-3'; COX-2: sense primer 5'-tctcaacactctcactac-3', antisense primer 5'-tcaggcttgatctgatcagc-3'; GAPDH: sense primer 5'-gaagtttctggcagc-3', antisense primer 5'-gggacctcacaactacag-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-ethylendiamine 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 administered (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 acetylated 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 protein 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.
3.3. PTS2 time-dependently inhibits LPS-induced iNOS up-regulation in RAW264.7 cells

We also detected duration of the anti-inflammatory activity of PTS2 in RAW264.7 cells. The cells were incubated with LPS (100 ng/ml), 3.0 μM PTS2 or both of them for 0–14 h, and then the cell lysates were subjected to Western blot analysis by using anti-iNOS antibody. As shown in Fig. 4A, LPS aroused a significant increase in iNOS protein level 8 h and 14 h after LPS treatment and PTS2 (3.0 μM) displayed more effective in inhibiting LPS-induced iNOS up-regulation at 8 h than 14 h time point after LPS treatment.

To evaluate the best time course of administration, we tested the effects of PTS2 on LPS-induced iNOS expression at four time points when PTS2 was administrated 0.5, 1, 2 and 4 h after LPS stimulation. The result from Western blot analysis showed that PTS2 inhibited LPS-induced up-regulation of iNOS protein at all time points and acted better when it was administrated with cells at 0.5 h than 1, 2 or 4 h time point after LPS treatment (Fig. 4B).

3.4. PTS2 inhibits LPS-increased NO production in RAW264.7 cells

NO is an important inflammatory product produced by iNOS and primarily involves in promoting inflammatory response [23]. We thus examined the effect of PTS2 on LPS-induced NO release from RAW264.7 cells by detecting changes of nitrite concentration in cell culture media. The cells were incubated with 3.0 μM PTS2 for 30 min after pretreatment with LPS (100 ng/ml). Twelve hours following PTS2 treatment, the culture media were collected and assayed for nitrite production. As shown in Fig. 5, PTS2 significantly inhibited LPS-induced NO release in RAW264.7 cells strongly suggesting an anti-inflammatory role of PTS2 in vitro.
3.5. PTS2 protects mice against LPS-induced mortality

We further evaluated the anti-inflammatory activity of PTS2 in the mouse model of endotoxin shock. For examining effects of PTS2 on septic shock, the mice were administrated with PTS2 (i.p.) 30 min after LPS (37.5 mg/kg) challenge and mortality was observed. As shown in Fig. 6, 90% mice died from endotoxin shock during 40 h after the injection (i.p.) of LPS. PTS2, at dose of 1, 2.5 or 5 mg/kg, raised the survival rate from 10% to 30%, 60% and 90%, respectively, in this animal model of endotoxin shock (Fig. 6A). To further observe the therapeutic effects of PTS2 on mortality of endotoxin shock, we administrated mice with 5 mg/kg of PTS2 4 h after LPS treatment. The data of Fig. 6B indicated that PTS2 raised the survival rate to 40%.

3.6. PTS2 decreases phosphorylation of STAT1 induced by LPS

Above experiments showed that PTS2 inhibited iNOS but not COX-2 expression at transcription level (Fig. 3). STAT1 has been reported to act as a transcription factor for increasing iNOS expression under LPS stimulation through binding with iNOS gene promoter sequence [24], whereas STAT pathway has a minor role in COX-2 expression [25,26]. In order to analyse the mechanism of PTS2 in inhibiting LPS-induced iNOS and COX-2 up-regulation, we further investigated the effect of PTS2 on STAT1 activation. RAW 246.7 cells were incubated with LPS (100 ng/ml), PTS2 (3.0 μM) or both of them, respectively, for 2 h or 5 h. The cell lysates were prepared for Western blot analysis. The results showed that STAT1 phosphorylation increased significantly in RAW246.7 cells 2 h after LPS stimulation and maintained at high level for 5 h, whereas PTS2 inhibited this LPS-induced STAT1 activation (Fig. 7). The basal level of STAT1 protein was not obviously affected by LPS and PTS2 treatment (Fig. 7). These results demonstrated that PTS2 significantly suppressed LPS-stimulated STAT1 phosphorylation, suggesting that PTS2 inhibited LPS-induced iNOS expression in macrophages by preventing STAT1 activation.

3.7. PTS2 does not influence LPS-induced MAPK and NF-κB activation

It has been well demonstrated that LPS activates MAPKs including ERK, JNK and p38 as well as NF-κB to produce various inflammatory mediators such as iNOS and COX-2 [27,28]. We thus evaluated the effects of PTS2 on LPS-induced JNK, ERK and p38 activation in RAW246.7 cells. The cells were incubated with LPS (100 ng/ml), PTS2 (3.0 μM) or both of them, respectively, for 0–4 h. Western blot analysis showed that MAPKs were activated by LPS in a time-dependent manner. Phosphorylation of JNK, ERK and p38 reached maximum 1 h after LPS stimulation and then gradually decreased. However, PTS2 did not affect LPS-stimulated JNK, ERK and p38 activation (Fig. 8). Exposure of cells to a variety of extracellular stimuli leads to the phosphorylation and ubiquitination of IκB-α proteins and their degradation by the proteasome, resulting NF-κB activation [29,30].
then investigated the effect of PTS2 on LPS-stimulated NF-κB activation in RAW246.7 cells via detecting IκB-α phosphorylation and degradation by Western blot analysis. The results showed that PTS2 showed no effect on LPS-induced IκB-α phosphorylation and degradation (Fig. 9A and B). These data demonstrated that PTS2 did not inhibit LPS-induced MAPK and NF-κB activation.

4. Discussion

Pyrithione (2-mercaptopyridine-1-oxide, PTO) has been noted for its highly bacteriocidal and fungicidal action for more than 50 years [31]. Compounds containing a –SH group are themselves oxidized to generate disulfide quickly. For PTO, such self-oxidation would result in the formation of the dimmer, dipyrithione (Fig. 1), which itself also possesses anti-bacterial and anti-fungal activity [32]. However, up till now, there is no report that demonstrates the anti-inflammatory activity of PTS2.

The cascades of events that lead to the highly fatal outcome of septic shock are believed to be triggered by the entrance of bacterial wall components, mainly the gram-negative endotoxin, into the systemic circulation [33]. A number of agents including glucocorticoid have been clinical used in terms of addressing the lethal effects of endotoxin. Glucocorticoids have been used as anti-inflammatory drugs for a long time, but frequent association of serious side effects, such as liver damage, cancers, stroke, growth stop, have been a long-standing dilemma in clinical steroid anti-inflammatory therapy. Non-steroidal anti-inflammatory drugs (NSAIDs) represent one of the most widely used drug classes including aspirin, indomethacin, phenylbutazone, etc. Moreover, new anti-inflammatory drugs are being discovered and developed based on their effects on signal transduction and as anti-cytokine agents [34]. Some pyridine derivates, such as pyroxin and sulfasalazine, have been known for their anti-inflammatory effects. PTS2 is one of the derivates from pyridine.

It has been well documented that LPS-induced endotoxic shock represents an adequate tool for understanding inflammatory processes [35]. The present study demonstrated that PTS2 is significantly effective in protecting mice from LPS-induced lethal shock suggesting its high anti-inflammatory activity. Pro-inflammatory cells are among the prime organizers of systemic inflammatory response syndrome and sepsis [36]. There is growing evidence that pro-inflammatory cytokines (e.g., interleukin-1 and tumor necrosis factor α) and mediators (e.g., NO and PGE2) play critical roles in the development and perpetuation of tissue inflammation and damage [37,38]. NO, synthesised by iNOS from l-arginine, is well known as an important mediator of acute and chronic inflammation. High concentration of NO combined with superoxide to form OONO-peroxynitrite ion which is responsible for severe cell damage and tissue destruction in inflammation. Our result that PTS2 significantly inhibited LPS-induced NO over-production by reducing iNOS over-expression in RAW246.7 cells demonstrated the anti-inflammatory effects of PTS2 on preventing the increase of pro-inflammatory factors stimulated by endotoxin.
PGE2 are chemicals that are important contributors to the inflammation that causes the pain, fever, swelling, and tenderness. COX, has two isoforms, COX-1 and COX-2. Inducible COX-2 is considered a pro-inflammatory enzyme and a target for the treatment of inflammatory diseases. Because COX-2 and iNOS were considered as two important pro-inflammatory enzymes, we also observed the changes of COX-2 after LPS stimulation and influence of PTS2. We found that PTS2 also significantly inhibited LPS-induced COX-2 protein increment in RAW 266.7 cells. These results strongly suggested that PTS2 could play an important role in protecting against cytotoxicity of NO and PGE2 in LPS-induced inflammatory responses and endotoxicemia.

In further analysis of the mechanism by which PTS2 inhibited LPS-induced iNOS and COX-2 expression, we found that though PTS2 decreased the protein level of both iNOS and COX-2, it suppressed LPS-induced iNOS but not COX-2 gene transcription. This result suggested that PTS2 decreased LPS-induced iNOS and COX-2 by different mechanism. The growing evidence have indicated that LPS stimulate iNOS and COX-2 gene expression by triggering MAPK and NF-κB activation and some inhibitors which block MAPK signaling and NF-κB activation prevent iNOS and COX-2 expression [39–41]. Thus, it is not surprising that PTS2 does not affect LPS-induced MAPK and NF-κB activation since it has no effect on LPS-induced increase of COX-2 mRNA level. As a signal transducer and activator of transcription, STAT1 could be phosphorylated on tyrosine residue 701 (Tyr-701) by LPS in a time-dependent manner [42]. A STAT1-binding GAS site in mouse iNOS gene promoter was found to be necessary for full expression of iNOS in LPS-stimulated RAW 264.7 macrophages [43]. Targeted disruption of the gene encoding STAT1 resulted in an inability to induce the accumulation of either protein or mRNA level of iNOS stimulated by LPS in macrophages [44]. However, STAT1 just plays a minor role in COX-2 transcription [25,26]. The results of the present study demonstrated that PTS2 apparently inhibited LPS-induced activation of STAT1 in RAW 266.7 macrophages suggesting that the inhibitory effect of PTS2 on STAT1 activation was a possible mechanism by which PTS2 suppressed LPS-induced iNOS but not COX-2 expression at mRNA level.

In response to inflammatory stimulation, when iNOS increased COX-2 also increased in a similar pattern [11]. The interaction of iNOS and COX-2 has been reported. Some anti-inflammatory agents which reducing NO production by inhibiting the expression of iNOS protein can further decrease COX-2 expression and activity [12,45]. The iNOS inhibitors prevent the formation of prostaglandins [46,47]. Furthermore, iNOS can bind and activate COX-2 [13]. The data from our study showed that unlike iNOS, LPS-induced COX-2 up-regulation was inhibited by PTS2 only at protein level. These results combined with the previous reports suggested that PTS2 might affect COX-2 protein level through altering iNOS expression.

In summary, our study demonstrated that PTS2 protected against endotoxic shock in mice and significantly inhibited
LPS-induced iNOS expression and NO production in macrophages. Meanwhile, PT2 also reduced LPS-induced increase of COX-2 protein level. Mechanistically, PT2 suppressed iNOS expression through reducing STAT1 phosphorylation and could possibly reduce LPS-induced increase of COX-2 protein level further. All these data demonstrated a novel activity of PT2 in anti-inflammatory and provided a possibility to develop PT2 as a candidate of anti-inflammatory agent in the future.

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References


