Fructose-1,6-diphosphate attenuates acute lung injury induced by lipopolysaccharide in mice

Hui Yin a,1, Xiao-bao Jin b,c,1, Quan Gong d, Heng Yang d, Li-yong Hu a, Fei-li Gong d, Jia-yong Zhu c,*

a Department of Microbiology and Immunology, Guangdong Pharmaceutical University, Guangzhou 510006, China
b School of Public Health and Tropical Medicine, Southern Medical University, Guangzhou 510515, China
c Institute of Pathogen Biology, Guangdong Pharmaceutical University, Guangzhou 510006, China
d Department of Immunology, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China

ARTICLE INFO

Article history:
Received 22 June 2008
Received in revised form 3 September 2008
Accepted 5 September 2008

Keywords:
Acute lung injury
Inflammation
Fructose-1,6-diphosphate
Lipopolysaccharide
NF-κB

ABSTRACT

Fructose-1,6-diphosphate (FDP), a high-energy glycolytic pathway intermediate, is reported to have a salutary effect in endotoxic shock and sepsis, but its underlying mechanism of action in inflammation is incompletely understood. In this study, our aim was to examine the function of FDP on acute lung injury (ALI) induced by lipopolysaccharide (LPS). We found that in vitro pretreatment with FDP remarkably repressed the production of TNF-α and IL-6 in murine alveolar macrophages MH-S exposed to LPS. In the mouse model of LPS-induced inflammatory lung injury, intravenous precondition of a single 400 mg/kg dose of FDP resulted in a significant reduction in LPS-mediated extravasation of Evans blue dye albumin, bronchoalveolar lavage fluid inflammatory content, and lung tissue myeloperoxidase activity (reflecting phagocyte infiltration). Furthermore, histopathologic examination indicated that alveolitis with inflammatory cells infiltration and alveolar hemorrhage in the alveolar space was less severe in the LPS-treated mice than in the mice treated by LPS alone at 24 h. Additionally, pretreatment with FDP markedly decreased the transcription of TNF-α, IL-6 and inducible NO synthase (iNOS), and suppressed the nuclear translocation of NF-κB in lung tissues in response to LPS challenge. These results thus suggested that FDP plays an anti-inflammatory role in LPS-mediated acute lung injury, possibly through abrogation of NF-κB activation.

© 2008 Elsevier B.V. All rights reserved.

1. Introduction

Acute lung injury (ALI) and its severest form, acute respiratory distress syndrome (ARDS), are associated with the development of multiple organ dysfunction syndrome (MODS), which plays a pivotal role in the death of patients with multiple transfusions, shock, sepsis and ischemia-reperfusion [1,2]. The pathogenesis of ALI/ARDS involves the disorders of oxidant/anti-oxidant and inflammation/anti-inflammation, the upregulation of adhesion molecules, and the increased production of chemokines. The histological change includes accumulation of a large variety of inflammatory cells, endothelial and alveolar epithelium cell apoptosis, increased pulmonary vascular permeability and the development of interstitial edema. Despite intense research and multiple diverse therapeutic trials, there is no effective treatment for it at present [1–3].

Fructose-1,6-diphosphate (FDP) is a naturally occurring high-energy glycolytic pathway intermediate and has been reported to exert substantial therapeutic effect in a variety of pathological situations [4]. It appears to decrease damage associated with ischemia, shock and toxic injury [5,6]. Experimental evidence indicates that the protective action of FDP in stress situations is mediated by its incorporation as an energy substrate and by prevention of critical alterations in cell membrane function. FDP treatment of animals subjected to endotoxin, traumatic, hemorrhagic, and compound 48/80-induced shock results in markedly reduced mortality [5–8]. In clinical patients with ARDS as a complication of sepsis, trauma, gastric aspiration, and other conditions, FDP treatment has been shown to improve pulmonary function and pulmonary and systemic hemodynamics [9]. Recent work has also demonstrated that FDP exerts protective effects on α-naphthylthiourea (ANTU) induced pulmonary edema [10]. It is well known that noncardiogenic pulmonary edema is caused by an acute inflammatory cascade in which the major participating factor is increased microvascular permeability. Damage to capillary endothelium is suggested to be caused largely by oxygen free radicals released from activated and sequestrated neutrophils on the lung. FDP has been found to effectively inhibit respiratory burst and oxyradical generation of activated neutrophils [2,11,12]. Increasing evidence shows that FDP possesses both in vitro and in vivo immunosuppressive properties, however the potential effect mechanism of FDP is not completely elucidated [13,14].

The aim of this study was to test the hypothesis that FDP, besides being a regulator of glycolytic metabolism, could play an anti-inflammatory role...
in LPS-induced acute lung injury. Moreover, the molecular mechanism of FDP might be involved in the modulation of inflammatory signaling pathway. By intravenous administration of FDP, we investigated its function on the LPS-induced murine ALI. We demonstrated here that FDP in vivo effectively attenuates lung tissue injury and suppresses the activation of inflammatory cascades. Furthermore, the protective effect of FDP in ALI was associated with down-regulation of NF-κB signaling pathway.

2. Materials and methods

2.1. Animals

Male BALB/c mice, 8–12 week old, were purchased from the Center of Experimental Animals of Chinese Academy of Medical Science and Center of Medical Experimental Animals of Hubei Province, and maintained at an animal facility under pathogen-free conditions. All studies involving mice were approved by the Huazhong University of Science and Technology Animal Care and Use Committee.

2.2. Culture, treatment and analysis of macrophages

Murine alveolar macrophages MH-S were plated in 24-well plates at 5 × 10^5 cells/ml in 1 ml complete medium (RPMI 1640 medium plus 5% FCS) overnight. Cells were then treated with 1 μM, 5 μM, 10 mM FDP (Sigma Chemical Co.), or medium for 3 h followed by stimulation with LPS (1 μg/ml). Culture supernatants were collected after 6 h and stored at −80 °C for cytokine analysis.

2.3. LPS-induced ALI model

After BALB/c mice were diethylether-anesthetized, 10 μg LPS (Escherichia coli O111:B4; Sigma) was administrated intranasally (i.n.) in 50 μl PBS to induce lung injury. Control mice were given 50 μl PBS i.n., without LPS as previously described [15]. FDP (400 mg/kg body weight) or vehicle (PBS) was administered intravenously (i.v.) 0.5 h prior to LPS administration. Bronchoalveolar lavage fluid (BALF) was performed by intratracheal instillation, and then the lungs were lavaged five times with 0.8 ml of sterile PBS. Recovered fluid from each sample was centrifuged (4 °C, 3000 rpm, 10 min) to pellet cells. The cell pellet was resuspended in PBS and counted with a hemocytometer.

2.4. Lung wet-to-dry weight (W/D) ratio

After euthanasia of mice, lungs were excised. Each lung was blotted dry, weighed, and then placed in an oven at 80 °C for 48 h to obtain the “dry” weight. The ratio of the wet lung to the dry lung was calculated to assess tissue edema.

2.5. Assessment of lung capillary leakage

The extravasation of Evans blue dye albumin (EBA; Sigma) into the tissue was used as an index of increased vascular permeability, as described previously [16]. Evans blue (20 mg/kg) was administered i.v. (1 ml/kg) via a tail vein, 30 min prior to termination of the experiment. The lung tissue was incubated in formamide (4 ml/200 g lung tissue; 24 h, 37 °C) and centrifuged at 5000 g for 30 min. The optical density of the supernatant was determined spectrophotometrically at 620 nm. EBA concentration was calculated against a standard curve and was expressed as micrograms of EBA/gram of tissue.

2.6. Myeloperoxidase assay

Neutrophil and macrophage parenchymal infiltration, reflected by myeloperoxidase (MPO) activity, was measured as described previously [17,18]. The lung tissues were homogenized and centrifuged (30000 g, 30 min at 4 °C). Pellets were resuspended in extraction buffer (50 mM potassium phosphate buffer containing 0.5% hexadecyl trimethylammonium bromide) and subjected to three cycles of freezing and thawing. The supernatants collected (13000 g, 15 min at 4 °C) were assayed for MPO activity by measuring the change in OD at 460 nm using kinetic readings over 3 min (200 μl sample with 800 μl reaction buffer containing 50 mM potassium phosphate buffer, 0.167 mg/ml of O-dianisidine dihydrochloride, and 0.0006% H₂O₂). Sample protein concentrations were determined (BCA assay), and the results are presented as MPO units per milligram of protein.

2.7. Histopathologic evaluation

Histopathologic evaluation was performed on animals that were not subjected to BAL. Lungs were inflated and fixed with 10% buffered formalin. Samples were embedded in paraffin, and then tissue sections were stained with H&E and examined under light microscope. The lung samples were assigned the following scores: 0 = normal; 1 = mild congestion, interstitial edema, and inflammatory infiltrate, with few red blood cells or inflammatory infiltrate in the alveolar spaces; 2 = moderate congestion and interstitial edema, partial filling of the alveoli with neutrophils, but without consolidation; 3 = marked congestion and interstitial edema with neutrophilic infiltrate nearly or completely filling the alveoli with consolidation.

2.8. SYBR Green real-time RT-PCR

Total RNA was extracted from the lung using the TRIzol reagent (Invitrogen) according to the manufacturer's instruction. After removal of potentially contaminating DNA with DNase I (Invitrogen), 3 μg of total RNA from each sample was used for reverse transcription with oligo (dT)

![Fig. 1](image-url)

**Fig. 1.** Effect of FDP on LPS-induced expression of TNF-α and IL-6 from MH-S cells. MH-S cells were pretreated with 1 mM, 5 mM, 10 mM FDP or medium alone for 3 h and then stimulated with LPS (1 μg/ml) for up to 6 h. Levels of TNF-α (A) and IL-6 (B) in the supernatant were measured by ELISA. Data are from three independent experiments and represent mean ± SEM. *P < 0.05 vs. the control group; #P < 0.05 vs. the LPS group.
18 primer and M-MuLV reverse transcriptase (Mbi Fermentas Inc., Burlington, USA) to generate first-strand cDNA. PCR mixture was prepared using Platinum® SYBR® Green qPCR supermix-UDG (Invitrogen) and using the primers as follows: TNF-α (forward 5′-CATCTTCTCAAATTC-GAGTGACAA-3′, reverse 5′-TGGGAGTAG ACAAGTACAACC-3′), IL-6 (forward 5′-GAGACTTCCCATCGTGACC-3′, reverse 5′-AAGTGCAT- CATCGTTGATCATACA-3′), iNOS (forward 5′-CAGCTGGGCTGTA-CAAACTT -3′, reverse 5′-CATTGGAAGTGAAGCGTTTCG-3′), and GAPDH (forward 5′-TTCACCACCATGGAGAAGGC-3′, reverse 5′-GGCATG- GACTGTGGTCATGA-3′). The PCR was conducted by using the following parameters: 50 °C for 2 min, 95 °C for 2 min, and 40 cycles of 95 °C for 15 s and 60 °C for 30 s on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, USA). Each gene expression was normalized with GAPDH mRNA content.

2.9. Electrophoretic mobility shift assay (EMSA)

NF-κB DNA binding activity was measured by EMSA using nuclear extracts from lung tissues. The biotin 5′ end-labeled duplex NF-κB oligonucleotide (5′-AGTTGAGGGGACTTTCCCAGGC-3′) was used as the probe, according to the LightShift Chemiluminescent EMSA (Pierce Biotechnology Inc., Rockford, USA). Incubations were done in a buffer containing 10 mM Tris (pH 7.6), 2.5% glycerol, 0.05% Nonidet P-40, and 50 ng/μl of poly (dl-dC). This reaction is then subjected to gel electrophoresis on a native (6%) polyacrylamide gel and transferred to a nylon membrane. The biotin end-labeled DNA is detected using the peroxidase-conjugated streptavidin system. For the cold competition assay, unlabeled probe was added in a 500-fold molar excess.

2.10. Statistical analysis

The histological score differences between groups were determined using the Kruskall–Wallis H test. Other results were analyzed by using Student’s t test or ANOVA. Data are expressed as the mean±SEM. Differences were considered to be statistically significant when P<0.05.

3. Results

3.1. FDP inhibits TNF-α and IL-6 production from LPS-exposed macrophages

To investigate the immunosuppressive role of FDP in inflammatory response, we examined its effect in vitro on the proinflammatory cytokine production from alveolar macrophage MH-S cells induced by LPS. MH-S cells were pretreated with FDP or medium alone for 3 h and

---

Fig. 2. Histologic assessment of the effect of FDP on LPS-induced lung inflammation and injury at 24 h. Lungs (n=3) from each experimental group were processed for histologic evaluation after hematoxylin and eosin staining. Histologic analysis of lung tissue obtained from control mice exposed to PBS identified preserved lung parenchymal architecture (A ×200, and D ×400). In contrast, mice exposed to intranasal LPS (B ×200, and E ×400) produced prominent neutrophils infiltration and alveolar hemorrhage (the arrow indicated). These features were dramatically decreased in mice treated with FDP prior to LPS challenge (C ×200, and F ×400).
3.2. In vivo FDP reduces LPS-induced murine lung injury

We next hypothesize that FDP could precondition against lung injury following LPS exposure through inactivation of inflammatory cells, especially alveolar macrophages and neutrophils. To test this, murine acute lung injury (ALI) model was established by intranasal instillation of LPS, which was assessed by histologic examination, measurement of BALF leukocytes, and lung MPO content. As shown in Fig. 2, LPS produced alveolar wall thickening, infiltration of neutrophils into the lung interstitium and alveolar space, as well as alveolar hemorrhage at 24 h. These findings were easily identified by blinded pathologic examination and were supported by quantification of neutrophils present in BALF (Fig. 3A) and in lung tissue from mice exposed to LPS (Fig. 2) when compared with control mice given PBS intranasally. Moreover, LPS-induced increases in tissue MPO activity, another reflection of lung parenchymal phagocyte infiltration, in contrast with control mice (Fig. 3B). Intravenous administration of a single dose of FDP prior to LPS exposure significantly reduced the inflammatory histologic changes produced by LPS in lung parenchyma at 24 h. FDP had no effect on lung tissue morphology in the absence of prior LPS challenge (data not shown). Consistent with these results, FDP also significantly decreased LPS-induced BALF neutrophilia compared with vehicle-treated mice, and attenuated both the neutrophil infiltration into the lungs of LPS-treated mice as well as increased tissue MPO activity observed in LPS-exposed mice (Fig. 3).

3.3. FDP attenuates pulmonary vascular permeability induced by LPS

LPS challenge produced a significant increase in capillary leakage, as shown by the lung wet/dry (W/D) ratio and the extravasation of EBA into lung parenchyma compared with control animals (Fig. 4). The administration of FDP intravenously did not significantly alter basal levels of lung wet/dry (W/D) ratio or EBA extravasation in control animals but significantly ameliorated the pulmonary vascular permeability in LPS-treated mice at 24 h after LPS challenge (Fig. 4).

3.4. Proinflammatory mediator levels in ALI are suppressed by FDP

Subsequently, we investigate the effect of FDP on the production of proinflammatory mediators such as TNF-α, IL-6 and iNOS, which is known to play a critical role in the pathophysiology of LPS-mediated ALI. Using real-time RT-PCR, we measured the expression of TNF-α, IL-6 and iNOS mRNA in lung tissues after LPS exposure. Compared with a control group, animals exposed to LPS resulted in an enhanced expression of TNF-α, IL-6 and iNOS mRNA at 3 h. In contrast, animals that pretreated with FDP showed minimal increases in TNF-α, IL-6 and iNOS mRNA.
iNOS mRNA levels compared with control mice when challenged by LPS (Fig. 5).

3.5. FDP downregulates inflammatory signaling pathway

NF-κB is a crucial transcription factor involved in signal transduction of a variety of extracellular stress stimuli [19]. It was shown that NF-κB is activated following LPS-induced acute lung injury and regulates both inflammatory and protective responses in the lung [20]. To investigate the possible molecular mechanism of FDP suppressing the gene expression of proinflammatory mediators, the activation of NF-κB was measured by electrophoretic mobility shift assay (EMSA). The results showed that NF-κB DNA binding activity was remarkably enhanced in the lung tissue at 3 h in LPS-challenged mice, when compared with control mice (Fig. 6). However, mice preadministered with FDP exhibited less NF-κB DNA binding activity. The specificity of the NF-κB bands was confirmed by cold competition analysis in the presence of excess unlabeled NF-κB consensus motif (Fig. 6).

4. Discussion

There is accumulating evidence showed that administration of FDP provides effective protection in endotoxic shock and ischemiareperfusion injury in lung, intestine, and kidney tissue [12,21,22]. It is well known that the aetiologies of acute lung injury (ALI) are complex and the mechanisms have been investigated extensively using different animal models. Moreover, intranasal instillation of LPS induces a more ideal experimental model of ALI, for it results in the lung injury without causing systemic inflammation and multi-organ failure [15]. However, the underlying mechanism by which FDP affords protection in inflammatory lung injury remains to be further clarified.

In the present study, we observed that preadministration of FDP dramatically reduced the pulmonary vascular leakage, W/D ratio, as well as leucocyte extravasation in BALF. The histological examination indicated that inflammatory cell infiltration and alveolar haemorrhage were less severe in FDP-treated mice than vehicle-treated group after LPS challenge. FDP also remarkably suppressed the transcription levels of proinflammatory mediators, such as TNF-α, IL-6 and inducible NO synthase (iNOS). Furthermore, we found that FDP effectively repressed the activation of transcription factor NF-κB, and this may be part of the mechanisms whereby FDP elicits its salutary effects.

Some studies proved that neutrophils sequestration into the pulmonary microvasculature and migration into the lung parenchyma is critical for host defense but also contributes to the development of ALI. Histopathologic examination of human lung specimens obtained early in the course of the disorder presented marked accumulation of neutrophils [23]. In addition to the involvement of the neutrophils in endotoxin-induced lung injury, the crucial role of the macrophages in this process has also been emphasized [24,25]. The characteristic pulmonary hypertension and increased microvascular permeability to endotoxin are abrogated if the animals are subjected to intravascular macrophage depletion or administration of detergent [24]. In this investigation, significant infiltration of neutrophils was observed in lung tissue by pathological examination in LPS-induced ALI. LPS caused an increase in the number of inflammatory cells in BALF. Furthermore, MPO activity, which reflects neutrophil and macrophage diapedesis, in lung tissue was significantly elevated after LPS exposure. In contrast, pretreatment with FDP markedly reduced the inflammatory histological changes in lung tissues produced by LPS challenge, as well as suppressed LPS-induced BALF neutrophilia and MPO activity (Fig. 3). These findings confirmed that the protective effect of FDP in ALI is related to attenuation of inflammatory cells sequestration and migration into the lung tissue.

Proinflammatory cytokines appear in the early phase of inflammatory response, and play a critical role in ALI and the acute respiratory distress syndrome (ARDS) [26]. The increased levels of TNF-α, IL-1β, and IL-6 in BALF are noted in ARDS patients, and the persistent elevation of proinflammatory cytokines in humans with ALI or sepsis has been associated with a worse outcome [27]. Moreover, instillation of IL-1β, TNF-α, or macrophage-inflammatory protein-2 (MIP-2) into the lungs leads to acute inflammatory injury [28]. Recently, FDP has been reported to exert protective effects in ischemia, shock and toxic injury associated with the down-regulation the production of proinflammatory cytokines. Evidence also showed that FDP preserved phagocytosis, besides inhibited the secretion of TNF-α and IL-1β, and the production of nitric oxide [22,29]. Several studies identified that FDP has an immunomodulatory function on the activity of the immune cells [14,30]. In vitro studies demonstrated that T lymphocyte proliferation and IL-2 expression are inhibited by FDP, whereas in vivo alone and in combination with CyA exhibited

---

**Fig. 5.** FDP suppresses the expression of proinflammatory mediators. Lung TNF-α (A), IL-6 (B) and iNOS (C) mRNA expression were measured in mice pretreated with FDP following LPS challenge at 3 h. Results were obtained using real-time RT-PCR and expressed as relative increase of mRNA expression compared with control animals. Data are presented as the mean±SEM (n=4–6 in each group). #P<vs. the LPS group.

*H. Yin et al. / International Immunopharmacology 8 (2008) 1842–1847*
prolonged cardiac allograft survival [30]. At the same time, our results suggested that pretreatment with FDP remarkably reduced the transcription of proinflammatory cytokines TNF-α, IL-1 and iNOS gene in lung tissues challenged by LPS.

To further illuminate the molecular mechanisms of FDP, which exerts anti-inflammatory signals leading to down-regulation of proinflammatory cytokines expression, we investigated the nuclear factor NF-κB signaling pathway in the lung tissue following LPS challenge. NF-κB is an important transcription factor required for the expression of a number of proinflammatory cytokines [19]. Furthermore, it has been demonstrated that the network of proinflammatory cytokines is regulated by the transcription factor NF-κB [20]. By using EMSA, we found that pretreatment with FDP could inhibit the NF-κB DNA binding activity. Moreover, this effect resulted in considerably suppression of TNF-α, IL-6 and iNOS genes which expression is modulated by NF-κB. Given that a large variety of proinflammatory cytokines regulated by NF-κB are primarily produced by inflammatory cells in the lung, our results consequently indicate that inactivation of inflammatory cells, especially alveolar macrophages and neutrophils, through inhibiting translocation of NF-κB into the nucleus is possibly the potential mechanism of FDP observed in this study.

In conclusion, our results revealed that the protective effect of FDP in ALL may be related to its suppression of NF-κB activation, and subsequently leads to a remarkable reduction in inflammatory cell infiltration, and proinflammatory cytokine expression in lung tissues. Therefore, these data strongly suggested FDP has potent anti-inflammatory actions that it may represent a novel strategy for the modulation of inflammatory response.

Acknowledgments

This work was supported by the National Development Program (973) for Key Basic Research (Grants 2007CB512402) of China, the National Natural Science Foundation of China (Grants 30671832), Ph.D. Research Foundation of Guangdong Pharmaceutical University (Grants 2007CJ06), and Medical and Scientific Research Foundation of Guangdong Province (A2008314).

References