P38 MAPK Pharmacological Inhibitor SB203580 Alleviates Total Parenteral Nutrition-Induced Loss of Intestinal Barrier Function but Promotes Hepatocyte Lipoapoptosis

Yong-Tao Xiao, Wei-Hui Yan, Yi Cao, Jun-Kai Yan, Wei Cai

Department of Pediatric Surgery, Xin Hua Hospital, School of Medicine, Shanghai Jiao Tong University; Shanghai Institute for Pediatric Research, Shanghai, Shanghai Key Laboratory of Pediatric Gastroenterology and Nutrition, Shanghai, China

Key Words
P38 MAPK • SB203580 • Total parenteral nutrition • Intestinal barrier • Lipoapoptosis

Abstract
Background & Aims: Our previous studies have provided evidence that p38 mitogen-activated protein kinase (MAPK) is involved in total parenteral nutrition (TPN)-associated complications, but its exact effects and mechanisms have not been fully understood. This study aimed to evaluate the roles of p38 MAPK inhibitor SB203580 in the TPN-induced loss of intestinal barrier function and liver disease. Methods: A rodent model of TPN was used to analyze the roles of SB203580 in TPN-associated complications. Intestinal barrier function was evaluated by transepithelial electrical resistance (TER) and paracellular permeability in Caco-2 cells. The palmitic acid (PA) was used to induce hepatic lipoapoptosis in vitro. The lipoapoptosis was detected using Caspase-3/7 and lipid staining. Results: In the present study, we showed that SB203580 treatment significantly suppressed TPN-mediated intestinal permeability in rats. SB203580 treatment significantly inhibited IL-1β-induced an increase in tight junction permeability of Caco-2 cells via repressing the p38/ATF-2 signaling. Unexpectedly, SB203580 treatment enhanced hepatic lipoapoptosis in the model of TPN. Palmitic acid (PA)-induced hepatic lipoapoptosis in human liver cells was significantly augmented by the SB203580 treatment. Conclusions: We demonstrate that the p38 MAPK inhibitor SB203580 ameliorates intestinal barrier function but promotes hepatic lipoapoptosis in model of TPN.

Introduction

The p38 mitogen-activated protein kinase (MAPK) family, including p38α, p38β, p38δ and p38γ, control a variety of cellular processes [1-7]. P38α is originally identified as a protein kinase implicated in stress and inflammatory responses [3, 6, 8]. Activation of p38
MAPK is induced by the MAPK kinases MKK3 and MKK6. Once p38 MAPK is activated, more than 100 proteins can be directly phosphorylated by p38α [9]. SB203580, a selective inhibitor of p38 MAPK that inhibits the catalytic activity of p38 MAPK by competitive binding to its ATP pocket, is effective in several disease models, including inflammation, arthritis diseases, septic shock and myocardial injury [10-12]. In these cases, p38 MAPK activation in key cell types was correlated with disease initiation and progression, and treatment with p38 MAPK inhibitor SB203580 blocked the activation of p38 MAPK and consequently alleviated disease severity. We recently reported that p38 MAPK activation was induced by total parenteral nutrition (TPN) [13], however, its exact roles and mechanisms remain unclear.

TPN is commonly used clinically for patients who are unable to tolerate enteral feedings, especially those with intestinal failure caused by short bowel syndrome, intestinal atresia, and other gastrointestinal malformations [14]. One of the major complications in TPN-infused patients is the loss of intestinal epithelial barrier function which may cause the penetration of luminal endotoxins or bacteria into the liver, leading to parenteral nutrition-associated liver disease (PNALD) [15-18]. This study aimed to investigate the roles of p38 MAPK in intestinal barrier loss and hepatic injury based on the model of TPN.

**Materials and Methods**

**Reagents**

Anti-phosphorylation-p38 kinase, p38, cleaved-caspase3, cleaved-PARP, phosphorylation-ATF-2, GAPDH and secondary antibodies conjugated to horseradish peroxidase were purchased from Cell Signaling Technology (Danvers, MA). Anti-PUMA and Bax antibodies were bought from Abcam (Bristol, UK). Palmitic acid, SB203580, FITC-Dextran and anti-MLCK antibody were obtained from Sigma-Aldrich (St. Louis, MO). Small interfering RNA (siRNA) of ATF-2 was obtained from GenePharma (Shanghai, China). Rat IL-1β ELISA was purchased from eBioscience (San Diego, CA).

**Total parenteral nutrition (TPN) model**

Three-week old male Sprague-Dawley rats (obtained from the Animal Experiment Center of the Chinese Academy of Science) were housed in individual cages and exposed to a 12-hour light-dark cycle for a week. Rats were randomized to sham (n=10), TPN (n=8) and TPN+SB203580 group (TPN+SB203580, n=6). The catheters for TPN were placed into the rats’ external jugular veins after anesthesia, and the rats were then infused with 30 mL/day for six days. Sham group received exactly same process and infused with saline and fed with standard chow ad libitum. As for TPN+SB203580 group, these TPN-rats were given SB203580 at concentration of 2 mg/kg/day as reported previously [19]. All experiments were approved by the Animal Care Committee of Xin Hua hospital, Shanghai Jiao Tong University. Composition of TPN solution is presented in Table 1.

**Measurement of intestinal permeability**

Intestinal permeability was tested with FITC-dextran in all rats. Briefly, FITC-dextran (dissolved in saline at a final concentration of 25 mg/mL) was given through gavage in a dose of 3 mL/100 g body weight at the day of sacrifice. Two hours after gavage, blood samples were collected by cardiac puncture and plasma concentration of FITC-dextran was determined using a fluorescence spectrometry with an excitation wavelength of 490 nm and an emission wavelength of 530 nm.

**Histological scoring and Immunohistochemistry (IHC) staining**

Intestinal tissues from each animal were stained with haematoxylin and eosin (H&E), and histological

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (mL)</th>
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<tbody>
<tr>
<td>8.5% amino acids</td>
<td>30</td>
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<tr>
<td>MCT/LCT emulsion</td>
<td>20.2</td>
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<tr>
<td>50% glucose</td>
<td>40</td>
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<tr>
<td>addamel</td>
<td>0.2</td>
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<tr>
<td>lipid-soluble vitamin</td>
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<tr>
<td>water-soluble vitamins</td>
<td>0.2</td>
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<tr>
<td>10% sodium chloride</td>
<td>3</td>
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<tr>
<td>10% potassium chloride</td>
<td>2</td>
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<tr>
<td>10% calcium gluconate</td>
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<td>phosphorus</td>
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changes were graded as previously described [20]. Briefly, histological scores were determined blindly based on sum of the epithelium and infiltration score. Epithelium score: 0=normal; 1=loss of goblet cells; 2=loss of goblet cells in large areas; 3=loss of crypts; 4=loss of crypts in large areas. Infiltration score: 0=normal; 1=infiltrate around crypt basis; 2=infiltrate reaching to muscularis mucosa; 3=extensive infiltration reaching the muscularis mucosa; 4=infiltration of the submucosa. Immunohistochemistry was performed using method of dianimesinidine (DAB) chromogen as described previously [21]. Briefly, paraffin-embedded tissues were incubated with xylol and descending concentrations of ethanol. Antigen retrieval was performed using citrate buffer, pH 6.0 or PH 8.0. Endogenous peroxidases were removed by incubation with 0.3% H2O2 for 15 minutes at room temperature (RT) and blocking was performed using 10% bovine serum albumin (BSA) for 1 hour at RT. Primary antibodies were then applied in an optimal concentration overnight in a wet chamber (Phosphorylated p38, dilution 1:100; Phosphorylated ATF-2, dilution 1:100; Bax, dilution 1:100; MLCK, dilution 1:50). Following incubation overnight at 4°C, the slides were rinsed in phosphate-buffered saline (PBS) and incubated with the secondary antibody for 1 hour at RT. Antibody binding was visualized by a liquid DAB Substrate Chromogen System (Dako, Glostrup, Denmark). The slides were rinsed in PBS and counterstained with hematoxylin. IHC images analysis was used software Image Pro Plus (Media Cybernetics) 10 fields/sample.

**Oil Red O staining and TUNEL test**

For H&E staining, the paraffin-embedded sections were sectioned at 4 μm and then stained. For Oil Red O staining, 4-μm-thick cryostat sections were stained with Oil Red O (sigma) for 30 min. After containing in 60% isopropanol, the sections were counterstained with hematoxylin. The images were acquired with a light microscope (Olympus, Tokyo, Japan), and the severity of steatosis was assessed by the proportion of Oil Red-positive hepatocytes (0= absent, 1 <=25%, 2=25-50%, 3 >=50%) as described previously [22]. TUNEL test (TdT-mediated dUTP nick end labeling) was performed using the "In Situ Cell Death Detection Kit" according to the manufacturers’ instructions (Roche Diagnostics).

**Cell culture and transfection**

Caco-2 cells (American Type Culture Collection, Manassas, VA), were maintained in a MEM medium with 20% fetal bovine serum (FBS). The human liver cell line L02 (purchased from shanghai Fuxiang Biotechnology Co., Ltd, China) were cultured in DMEM supplemented with 10% FBS at 37°C with 5% CO2 in a humidified atmosphere. The human primary liver cells were bought from the Research Institute for Liver Disease (Shanghai) Co., Ltd. Transient transfections with ATF-2 siRNA was purchased from GenePharma Inc (Shanghai, China), and the sequences are 5’-CCUCUUGCAACACCUAUCATT-3’ , 5’-CGAGUCUAAUGAGAAUGATT-3’, and 5’-CCUGUGAAUAUGAGUGAATT-3’.

**Measurement of mitochondrial membrane potential**

L02 cells were seeded into a 96-well plate at a density of 0.5 × 104 cells/well. On the next day, the medium was replaced with DMEM containing 400 μM palmitate (PA) and/or 20 μM SB203580, and mitochondrial membrane potentials were detected 16 hours later using a JC-1 Mitochondrial Membrane Potential Probe (Thermo Fisher Scientific, Waltham, MA). Labeled cells were observed using an inverted fluorescence microscope (TMS, Nikon, Tokyo, Japan). The ratio of JC1-monomers (excitation at 485 nm and emission at 535 nm) to J-aggregates (excitation at 560 nm and emission at 595 nm) was used as an indicator of cell apoptosis.

**Caspase-3/7 and lipid staining**

L02 cells and human liver primary cells were seeded into a 96-well plate and treated with 400 μM PA and/or 20 μM SB203580 for 12-48 h. The apoptotic cells were detected with CellEvent™ Caspase-3/7 Green Ready Probes® Reagent (Thermo Fisher Scientific, Waltham, MA), according to the manufacturers’ protocol. For lipid staining, the cells were analyzed with HCS LipidTOX™ Green Neutral Lipid Stain (Thermo Fisher Scientific, Waltham, MA), according to the manufacturers protocol.

**Western blotting**

Western blotting was performed as previously described [1]. For assessment of protein expression in Caco-2 cells, Caco-2 monolayers were treated with IL-1β (10 ng/ml), SB203580 or ATF-2 siRNA transfection for varying time periods. At the end of the experimental period, Caco-2 monolayers were immediately rinsed
with ice-cold PBS, and cells were lysed with lysis buffer. Aliquots of 40 μg protein/well were separated on 4-12% SDS-polyacrylamide gels and transferred onto nitrocellulose membrane using a dry blotting system (iBLOT system, Invitrogen Inc). After blocking with PBS containing 5% nonfat milk at room temperature for 1 hour, membranes were incubated with the primary antibodies overnight at 4°C. The membranes were washed three times with PBS, 0.1% Tween-20, and then incubated with secondary antibodies. After final washes with PBS, 0.1% Tween-20, the signals were detected using ECL chemiluminescence reagents (Pierce, Inc). The primary antibodies of phosphorylated p38, total p38, phosphorylated ATF-2, Bax, PUMA, Cleaved-Caspase3, Cleaved-PRAP, MLCK and GAPDH were performed in this study.

**Transepithelial electrical resistance (TER)**

SB203580 (10 μM) was added to Caco-2 monolayers 1 h prior to the IL-1β (10 ng/mL) treatment. The effect of SB203580 on the IL-1β-induced increase in Caco-2 tight junction permeability was measured at 48 h following IL-1β treatment. Transepithelial electrical resistance (TER) of the filter-grown Caco-2 cells was measured using an epithelial voltohmmeter (The Millicell® Electrical Resistance System, Millipore Corp, MA, USA) as described previously. The relative TER in various treatment groups was calculated as a percentage of the control Caco-2 monolayers. The control relative TER was set to 100%.

**Paracellular permeability analysis**

Paracellular permeability was determined by measuring apical to basolateral flux of fluoresceinated dextran (FD4, FITC-dextran; Sigma) using a modification of previously described method [23]. Briefly, confluent epithelial monolayers on 0.4μm-pore size Transwells were washed twice with Hanks’s balanced salt solution. FD-4 (1 mg/mL) was added apically at time 0, and samples (50 μL) were taken from the basal compartment at 30, 60, 90, and 120 min. The concentration of FITC-dextran was determined using a fluorescence plate reader with an excitation wavelength of 490 nm and an emission wavelength of 530 nm.

**Statistical analysis**

Data are presented as mean ± SD. For comparisons of different groups, statistical significance was determined based on the Student’s t test. P values <0.05 were considered statistically significant.

**Results**

**P38 MAPK activation is inhibited by SB203580 treatment in the model of TPN**

p38α, one of four known isoforms of p38 MAPK, is ubiquitously expressed and susceptible to dual phosphorylation at its Thr180 and Tyr182 residues for the activation of p38 MAPK. We here showed that TPN treatment significantly induced the expression of phosphorylated-p38 MAPK (Thr180 and Tyr182) in jejunal villus and crypt (Fig. 1A). Activated p38 MAP kinase has been shown to phosphorylate and activate MAPKAP kinase 2 and futher to phosphorylate the transcription factor ATF-2. As expected, the number of phosphorylated-ATF-2 (Thr71)-positive cells was markedly increased after 6 days of TPN administration (Fig. 1A). It is reported that SB203580 could suppress the phosphorylation of the p38 MAP kinase in vitro and in vivo [11, 24]. As shown in Figure 1A, SB203580 treatment completely prevented the TPN-induced activation of p38 MAPK (Fig. 1A). Additionally, it was also observed that expression of myosin L chain kinase (MLCK) significantly increased in rats after giving TPN (Fig. 1A).

**SB203580 treatment prevents TPN-induced intestinal barrier loss in vivo**

P38 MAPK is thought to play an essential role in barrier dysfunction in response to inflammatory cytokines stimulation [25]. We here firstly showed that treatment with SB203580 dramatically reduced the severity of TPN-induced intestinal injury through blinded scoring of intestinal sections (Fig.1A). We also investigated the role of SB203580 treatment in TPN-induced intestinal permeability by assessing the concentration of FITC-conjugated dextran in blood. Plasma levels of FITC-dextran were significantly increased in TPN group. In contrast, SB203580 treatment dramatically reduced the levels of plasma...
Interleukin-1β (IL-1β) is a prototypical multifunctional cytokine that plays an important role in intestinal inflammation [26]. We here detected the serum levels of IL-1β in all rats. As shown in Figure 1C, the serum levels of IL-1β were significantly increased in TPN group when compared to sham group, which was evidently reduced by SB203580 treatment (Fig. 1C). However, there were not statistical differences in serum levels of IL-1β in the SB203580 plus TPN group compared to the TPN group (Fig. 1C).
SB203580 treatment inhibits IL-1β-induced increase in intestinal permeability in vitro

We next examined the involvement of the p38 MAPK in IL-1β-induced increase in intestinal permeability in vitro. As shown in Figure 2A, the effect of IL-1β on p38 MAPK signaling was determined by p38 MAPK and ATF-2 phosphorylation. IL-1β treatment at physiological concentration (10 ng/ml) with various time caused a significantly increase in p38 MAPK and ATF-2 phosphorylation in Caco-2 cells (Fig. 2A). In addition, IL-1β treatment also induced increase in MLCK expression (Fig. 2A). Blocking activation of p38 MAPK signaling with SB203580 treatment or ATF-2 silence exhibited significant reduction in MLCK expression, suggesting the requirement of p38 MAPK in the IL-1β-induced MLCK expression. The effects of IL-1β on the permeability of Caco-2 cells were determined by measuring TER.
and paracellular permeability. IL-1β treatment caused a significant decrease in TER and a markedly increase in FITC-dextran flux, and these effects were significantly attenuated by SB203580 treatment or ATF-2 knockdown (Fig. 2B, C).

SB203580 treatment increases the TPN-associated hepatic lipoapoptosis in vivo

Unexpectedly, it was observed that SB203580 treatment aggrevated hepatic steatosis in TPN-treated rats. To fully characterize this observation, we examined the effects of SB203580 treatment on levels of liver triacylglycerol contents and plasma lipids. As shown in Figure 3A, the Oil Red O staining showed that the fat droplets were elevated in TPN rats compared with sham rats (Fig. 3A). Moreover, rats in the group of TPN plus SB203580 showed a further increase in fat droplets (Fig. 3A). Consistent with these results, the serum levels of cholesterol and triacylglycerol were higher in the group of TPN plus SB203580 treatment than those in the TPN group (Table 2).

Bax is a known mediator of mitochondrial dysfunction, which is required for induction of hepatic lipoapoptosis [27]. We demonstrated that treatment with SB203580 significantly inhibited the p38 activation but evidently promoted the expression of hepatic Bax (Fig. 3B). Furthermore, TUNEL test confirmed SB203580 treatment could increase hepatic apoptosis (Fig. 3B).

SB203580 treatment promotes palmitic acid-mediated hepatic lipoapoptosis in vitro

We further examined the effect of p38 inhibition on palmitic acid (PA)-mediated lipoapoptosis in human liver cell line L02 and human primary liver cells with SB203580 treatment. We showed that PA stimulated p38 MAPK activation and SB203580 effectively reduced PA-induced p38 MAPK activity, by assessing phosphorylation of p38 MAPK. As shown in Figure 4, the images showed that the SB203580 treatment increased PA-induced lipid and fat droplets in human liver cell line L02 and human primary liver cells (Fig. 4A, B). The loss of mitochondrial membrane permeability (MMP) is a prominent feature of
the mitochondrial pathway of apoptosis. The MMP was much lower in cells with PA plus SB203580 treatment compared to cells with PA addition (Fig. 4A). Since caspase3/7 activation mediates the apoptotic phenotype, we next confirmed that the effect of SB203580 on PA-induced apoptosis was caspase dependent, as it further increased caspase 3/7 (Fig. 4A). Moreover, western-blot analysis showed that SB203580 treatment increased the apoptotic makers, including PUMA, Bax, cleaved-caspase3 and cleaved-PARP expression (Fig. 5).

**Discussion**

In the present study, we were to explore the role of p38 MAPK in TPN-associated loss of intestinal epithelial barrier function and liver injury. To this end, we firstly pharmacologically inhibited p38 MAPK in the rat model of TPN using the specific p38 MAPK inhibitor SB203580. In agreement with the previous studies [10, 11], we showed that SB203580 was a highly selective inhibitor of p38 MAPK that could significantly inhibit TPN-induced p38 MAPK activation. ATF-2 is a direct substrate of p38 MAPK and is activated by phosphorylation at Thr69 and Thr71 [28, 29]. We here observed that TPN-induced phosphorylation of ATF-2 (Thr71) was nearly suppressed by SB203580 treatment. We secondly determine the roles of SB203580 in TPN-induced increase in intestinal permeability. In line with previous reports [30-32], we found that plasma FITC-dextran levels increased significantly following...
TPN administration. After treatment with SB203580, the concentration of plasma FITC-dextran declined markedly, suggesting an important role of p38 MAPK in TPN-induced loss of intestinal barrier function. However, the involved mechanisms have not been completely established. IL-1β, a prototypical multifunctional cytokine, plays an important role in intestinal inflammation and causes increase in intestinal epithelial permeability both in vivo and in vitro [33-35]. We here showed that TPN administration significantly increased levels of serum IL-1β. It was noted that SB203580 treatment could reduce TPN-induced increase in IL-1β expression. Interestingly, there are not statistical differences in serum levels of IL-1β in the group of TPN plus SB203580 treatment compared to the TPN group. It has been reported that p38 MAPK activation is important to IL-1β production in serveral cell types [36-39]. In turn, numerous studies indicated that the activation of nuclear factor-kappaB was essential to the production of IL-1β [40]. Thus, this phenomenon was attibuted to the possibility that the production of IL-1β was, in part, due to p38 MAPK activation. Additionally, p38 MAPK pathway is essential to the IL-1β-mediated immune responses and loss of intestinal barrier function [41, 42]. In this study, we indicated that IL-1β could significantly increase the tight junction permeability in Caco-2 cells. Simultaneously, IL-1β stimulated the activation of p38 MAPK pathway. Previous studies suggested that IL-1β-induced increase tight junction permeability in Caco-2 cells was mediated by an increase in MLCK expression [33, 35, 42]. In combination, activated ATF-2 can attach to its binding motif on the MLCK promoter region and enhance the MLCK promoter activity and gene transcription [42]. Indeed, we indicated that silence of ATF-2 with specific siRNA significantly inhibited the expression of MLCK and further suppressed the IL-1β-induced increase in tight junction permeability.

Unexpectedly, we observed that SB203580 treatment increased hepatic steatosis and apoptosis. Our results showed that levels of triacylglycerol and apoptotic marker Bax expression were significantly increased in the rats with TPN and/or SB203580 treatment. Xiong et al. recently reported that blockade of p38 MAPK could lead to hypertriglyceridemia and fatty liver [43], which suggested that the inhibitory role of p38 MAPK in hepatic lipogenesis may be pivotal for maintaining plasma glucose levels and limiting lipid synthesis in liver when excess calories, particularly fats, are ingested. Saturated free fatty acids can induce hepatocyte lipoapoptosis. We here indicated that PA treatment could increase the number of the lipid droplets and the expression of the apoptotic marker Bax, Caspase3/7 and PUMA in the liver cells, and these effects were significantly aggravated by the SB203580 treatment. Although the precise cellular and molecular mechanisms of hepatocyte lipoapoptosis have not been fully elucidated, PUMA knockdown has been shown to markedly reduce hepatic lipoapoptosis [44]. Therefore, we suppose that PUMA maybe an important death mediator in the p38 MAPK inhibition-induced hepatic lipoapoptosis.

In conclusion, these evidence revealed that p38 inhibition alleviates TPN-induced increase in intestinal permeability but promotes the hepatic lipoapoptosis.
Abbreviations

PNALD, (parenteral nutrition-associated liver disease); MAPK, (mitogen-activated protein kinase Introduction); TPN, (total parenteral nutrition); ALT, (alanine aminotransferase); AST, (aspartate aminotransferase).

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Disclosure Statement

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