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Role of p38 MAPK in Burn-Induced Intestinal Barrier Breakdown[1]

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Abstract

Background—Severe burn results in intestinal barrier breakdown, which may lead to the generation of a systemic inflammatory response and distant organ injury. Intestinal barrier integrity is regulated, in part, by the tight junction protein myosin light chain kinase (MLCK). Previous studies in cell culture have shown that activation of p38 MAPK plays an important role in modulating intestinal barrier function. We hypothesized that (1) severe burn up-regulates p38 MAPK activation and results in increased intestinal permeability via augmented expression of MLCK, and (2) inhibition of p38 MAPK will prevent the burn-induced increase in MLCK expression, resulting in improved intestinal barrier integrity.

Materials and Methods—Male balb/c mice were subjected to a 30% total body surface area (TBSA) full thickness steam burn, then randomized to receive an intraperitoneal injection of a p38 MAPK inhibitor (SB203580, 25 mg/kg) or vehicle. In vivo intestinal permeability to 4kDa FITC-Dextran was measured. Expression of phosphorylated p38 MAPK, total p38 MAPK, MLCK, and phosphorylated MLC from intestinal extracts was assessed by immunoblotting.

Results—Severe burn increased intestinal permeability, which was associated with activation of p38 MAPK, and increased expression of MLCK. Treatment with SB203580 significantly attenuated burn-induced intestinal permeability (212 μg/mL *versus* 81 μg/mL, $P < 0.05$), and decreased expression of intestinal MLCK resulting in decreased phosphorylation of MLC.

Conclusion—p38 MAPK plays an important role in regulating burn-induced intestinal permeability through activation of MLCK. Inhibition of p38 MAPK may be an important therapeutic target aimed at attenuating intestinal barrier breakdown by preventing the burn-induced alterations in tight junction proteins.

Keywords

intestinal permeability; p38 MAPK; myosin light chain kinase; inflammation; intestine; burn; intestinal barrier; tight junction; gut

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INTRODUCTION

The intestinal epithelium forms a physical barrier that is responsible for protecting the host against potentially dangerous luminal contents. While intestinal epithelial cells form a barrier within the lumen of the intestine, the paracellular space between adjacent epithelial cells represents a potential space for the passage of molecules. The intestinal tight junction proteins sit at the apical edge of the intestinal epithelium and regulate gut barrier function by restricting the movement of luminal contents through this paracellular space [1]. These tight junction proteins can be modulated by inflammatory stimuli, resulting in loss of the intestinal barrier [2].

Gut barrier failure plays an important role in the development of the systemic inflammatory response (SIRS) and distant organ injury that is responsible for significant morbidity and mortality in severely injured patients [3]. The intestinal inflammatory response generated following injury can result in the production of pro-inflammatory cytokines in the gut, which are then spread systemically *via* the intestinal lymph [4]. We have previously shown that severe injury results in the loss of tight junction proteins, which was associated with increased intestinal permeability and intestinal cytokine generation [5]. Understanding the signaling mechanisms that modulate tight junction breakdown following injury may be important in developing therapeutics aimed at limiting the intestinal inflammatory response.

Inflammatory signaling within the intestinal epithelial cell leads to modulation of intestinal barrier function *via* activation of myosin light chain kinase (MLCK), a key regulator of tight junction permeability. Increased MLCK protein expression leads to increased permeability across the tight junction, and has previously been shown to be modulated *via* the Nuclear factor-kappa B (NF-κB) signaling cascade [6]. We have recently demonstrated in an *in vivo* model that severe burn injury increases intestinal MLCK protein expression, which is associated with increased NFκB nuclear translocation [7]. Inflammatory signaling *via* p38 mitogen-activated protein kinase (MAPK) may also be an important regulator of intestinal permeability. Recent *in vitro* studies have shown that pro-inflammatory cytokines increase phosphorylation of intestinal p38 MAPK, with pharmacologic inhibition of p38 MAPK activation improving intestinal barrier integrity [8].

In this study, we further explored the effects of severe injury on intestinal barrier function using an *in vivo* model of severe burn injury. We hypothesized that severe injury would increase phosphorylation of intestinal p38 MAPK, resulting in intestinal permeability that was associated with an increase in MLCK protein expression.

MATERIALS AND METHODS

Burn Model of Injury

All animal experiments were approved by the University of California San Diego Institutional Animal Care and Use Committee. Male balb/c mice weighing 20 to 24 g were purchased from Jackson Laboratory (Sacramento, CA). Animals were anesthetized with inhaled isoflurane. Following induction of general anesthesia, the dorsal fur was clipped using an electric clipper. Animals were then placed into a template constructed to estimate a

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30% TBSA based on the Walker-Mason burn model and underwent a 7 s steam burn. Immediately following burn, animals received an intraperitoneal injection of SB203580 (25 mg/kg; LC Laboratories, Woburn, MA) diluted in dimethyl sulfoxide (DMSO) or an equal volume of DMSO alone. The dose of SB203580 (25 mg/kg) was chosen based on a previous dose response curve published by Badger *et al.*, demonstrating that this dose resulted in a greater than 70% decrease in inflammatory cytokine levels after injection of endotoxin [9]. Animals also received a subcutaneous injection of 1.5 mL normal saline containing buprenorphine for fluid resuscitation and pain control. Animals were returned to their cage following burn injury, recovered from anesthesia, and were allowed access to food and water *ad libitum*. Sham animals were placed under general anesthesia, underwent dorsal fur clipping, and received a subcutaneous injection of normal saline with buprenorphine but were not burned.

Tissue Harvest

Animals were sacrificed at 2 h following injury. Segments of distal ileum were immediately snap-frozen in liquid nitrogen and stored at −70 °C for later analysis. Samples of distal ileum were also preserved in 10% Formalin for histologic analysis.

Intestinal Permeability Assay

Two hours following severe burn, animals (*n* 4 per group) were again anesthetized with inhaled isoflurane for measurement of intestinal permeability. A midline laparotomy was performed in order to isolate a 5 cm segment of distal small intestine between silk ties. A 200 μL solution containing 25 mg of FITC-dextran (4 kDa, Sigma, St. Louis, MO) in phosphate buffered saline (PBS) was injected into the lumen of the isolated segment of intestine. The small bowel was then returned to the abdominal cavity and the abdominal wall closed. Animals were maintained under general anesthesia until 30 min following injection of FITC-dextran, when a cardiac puncture was performed to obtain a specimen of systemic blood. Blood was kept on ice until serum was obtained by centrifuging the blood at 10,000 *g* for 10 min. The plasma was analyzed for FITC-dextran using a fluorescence spectrophotometer (SpectraMax M5; Molecular Devices, Sunnyvale, CA). The concentration of FITC-dextran was obtained by comparing the fluorescence of the plasma samples to a standard curve of known concentrations of FITC-dextran diluted in mouse serum.

Histologic Evaluation

Distal ileum obtained 2 h following burn (*n* 3 animals per group) was stored in 10% formalin and embedded in paraffin blocks using an automated processor. Sections of gut were cut 7 μm thick, placed onto glass slides, and stained with hematoxylin-eosin. The stained slides were viewed with an Olympus IX70 light microscope (Olympus, Melville, NY) at ×20 magnification. Images were obtained using Q-imaging software (Surrey, British Columbia, Canada).

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Immunoblotting

Samples of distal ileum obtained 2 h following burn were homogenized in a 500 μL solution containing ice-cold tissue protein extraction reagent (TPER) with 1% protease inhibitor and 1% phosphatase inhibitor (Pierce Biotechnology, Rockford, IL). The homogenates were then centrifuged at 10,000 *g* for 5 min, and the supernatant was collected. Protein concentration of each sample was determined using the bicinchoninic acid protein assay kit (Pierce) using the microplate reader protocol. Western blots were performed by separating proteins with SDS-polyacrylamide gel electrophoresis using 8%–16% Tris-glycine polyacrylamide gels (Invitrogen, Carlsbad, CA). Proteins were transferred onto nitrocellulose membranes (Invitrogen) and blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline (TBS)/Tween 20. Membranes were incubated in primary antibody prepared in 5% BSA (1:500) for phosphorylated p38 MAPK, p38 MAPK, phosphorylated myosin light chain (MLC), β actin (Cell Signaling, Danvers, MA), or MLCK (Sigma). Membranes were incubated with secondary antibody, horseradish peroxidase-linked anti-rabbit IgG (1:2000; Cell Signaling) prepared in 5% BSA blocking solution. Membranes were incubated with Pierce Supersignal West Pico Chemiluminescent Kit for 3 min prior to detection of luminescence using the Xenogen IVIS Lumina imaging system (Caliper Life Sciences, Mountain View, CA). Mean pixel density of each gel was estimated using UN-SCAN-IT Gel Digitizing software (Silk Scientific, Orem, UT). The relative band density of each band was calculated by dividing the pixel density by the mean pixel density of the sham samples.

Statistical Analysis

Values are expressed as the mean ± SEM of *n* samples, where *n* represents the number of animals in each experimental group. The statistical significance between groups was determined using analysis of variance (ANOVA) with Bonferroni correction using SPSS Statistics (Chicago, IL). A $P < 0.05$ was considered statistically significant.

RESULTS

Burn-induced Intestinal Permeability

In vivo intestinal permeability was assessed by measuring the concentration of FITC-dextran in the systemic circulation following intraluminal injection of the fluorescent tracer (Fig. 1). Intestinal permeability to 4 kDa FITC-dextran is significantly increased 2 h following 30% TBSA full thickness burn compared to sham (212 μg/mL *versus* 25 μg/mL, $P < 0.03$). Treatment with the p38 MAPK inhibitor SB203580 attenuated the burn-induced increase in intestinal permeability (81 μg/mL , $P < 0.05$ *versus* Burn/Vehicle).

Intestinal Histology

Sections of distal ileum harvested 2 h following severe burn show early signs of histologic injury characterized by evidence of mucosal ulceration at the villous tips and signs of early inflammatory cell infiltration (Fig. 2). Villous height remains normal in burned animals. Sections of gut from animals treated with the p38 MAPK inhibitor SB203580 appear similar to sham, with normal villous architecture without mucosal ulceration.

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Intestinal p38 MAPK Activation

Activation of intestinal p38 MAPK was assessed 2 h following injury. Severe burn injury results in a 5-fold increase in phosphorylation of p38 MAPK over sham ($P < 0.05$, Fig. 3). Intraperitoneal injection of the p38 MAPK inhibitor SB203580 prevented the burn-induced phosphorylation of p38 MAPK. There was no change in p38 MAPK protein levels in any group.

Burn-induced Myosin Light Chain Kinase Expression

Intestinal MLCK protein expression was assessed using Western blot 2 h following 30% TBSA burn (Fig. 4A and B). There was nearly a 6-fold increase in intestinal MLCK levels in burned animals compared with sham ($P < 0.03$). Treatment with the p38 MAPK inhibitor prevented the burn-induced increase in MLCK expression ($P < 0.03$ *versus* Burn/Vehicle), with levels similar to sham.

Phosphorylation of Intestinal Myosin Light Chain

Activation of MLCK results in phosphorylation of MLC. Therefore, we assessed intestinal MLC phosphorylation to confirm the effects of changes in MLCK protein expression. Burn injury increased phosphorylation of MLC by 3-fold compared with sham ($P < 0.001$, Fig. 4C and D). Injection of SB203580 immediately following burn prevented the burn-induced phosphorylation of MLC ($P < 0.01$ *versus* Burn/Vehicle).

DISCUSSION

In this study, we demonstrate the importance of p38 MAPK activation on intestinal barrier breakdown following severe injury. This is the first *in vivo* study, to our knowledge, to demonstrate that preventing phosphorylation of intestinal p38 MAPK results in improved intestinal barrier integrity. Intraperitoneal injection of SB203580 decreased intestinal permeability to a 4 kDa fluorescent tracer. These results extend the recent *in vitro* work of Wang *et al.*, which demonstrates the importance of MAPK signaling on intestinal barrier integrity [8].

Inhibition of p38MAPK following thermal injury also attenuated histologic gut injury. There was early evidence of intestinal injury in animals 2 h following burn, suggesting that intestinal permeability occurs prior to significant changes in gut architecture. In previous studies using this model, we have seen marked changes in histologic appearance of the gut at 6 h following burn [5].

Attenuation of intestinal permeability with the p38 MAPK inhibitor was associated with decreased intestinal MLCK expression. Severe burn injury resulted in a 6-fold increase in MLCK protein in animals injected with vehicle alone, resulting in increased phosphorylation of MLC. Injection of SB203580 immediately following burn significantly decreased intestinal MLCK expression and MLC phosphorylation. As expected, this decrease in MLC phosphorylation was associated with decreased intestinal permeability to FITC-dextran.

The intestinal tight junction plays a key role in determining intestinal barrier function by controlling the movement of luminal contents through the paracellular space between

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adjacent epithelial cells [10]. MLCK is an important regulator of tight junction permeability through its ability to phosphorylate MLC. Once phosphorylated, MLC causes contraction of the perijunctional actin cytoskeleton causing loss of the tight junction barrier [11, 12]. *In vitro* studies have shown that increased expression of MLCK leads to increased phosphorylation of MLC and elevated intestinal permeability [13]. Preventing activation of MLCK may have important therapeutic implications, as MLCK inhibitors have been shown to decrease phosphorylation of MLC and restore intestinal barrier integrity [14].

Increased MLCK protein expression and increased intestinal epithelial permeability have been demonstrated in response to stimulation with TNF-α [15]. Graham *et al.* has characterized the promoter region responsible for the TNF-α-induced up-regulation of MLCK transcription in the intestinal epithelium [16]. They identified a MLCK promoter region which contained binding sites for the transcriptional factors activator protein-1 (AP-1) and NF-κB. They demonstrated that binding of these transcriptional factors to the MLCK promoter region increased the transcription of MLCK. Signaling *via* p38 MAPK is known to affect gene transcription *via* AP-1[17]. Inhibition of p38 MAPK in intestinal cells has also been shown to reduce the DNA binding of the AP-1 transcriptional factor *in vitro* [18].

We have previously demonstrated that this thermal injury model increases both circulating and intestinal levels of TNF-α [5]. By preventing phosphorylation of p38 MAPK, there may be decreased activation of AP-1, which may account for the decrease in MLCK protein expression seen in these animals. Further studies are needed to definitively link the phosphorylation of p38 MAPK to DNA binding of AP-1, and subsequent MLCK-induced increases in intestinal permeability. Signaling *via* the NF-κB signaling pathway is also clearly important in the regulation of MLCK expression. While we have previously shown that severe burn increases NF-κB nuclear translocation, it is unknown what the effects of p38 MAPK inhibition are on the ability of NF-κB to cause changes in the intestinal tight junction.

To date, this is the first series of *in vivo* experiments to document the role of p38 MAPK in burn-induced intestinal barrier injury. We demonstrated that pharmacologic inhibition with a p38 MAPK inhibitor improved intestinal barrier integrity, which was associated with decreased activation of the tight junction protein MLCK. Inhibition of p38 MAPK may be an important therapeutic target aimed at preventing intestinal permeability following severe injury. Preventing intestinal barrier breakdown may decrease the systemic sequelae associated with the intestinal inflammatory response, and result in improved outcomes in severely injured trauma and burn patients.

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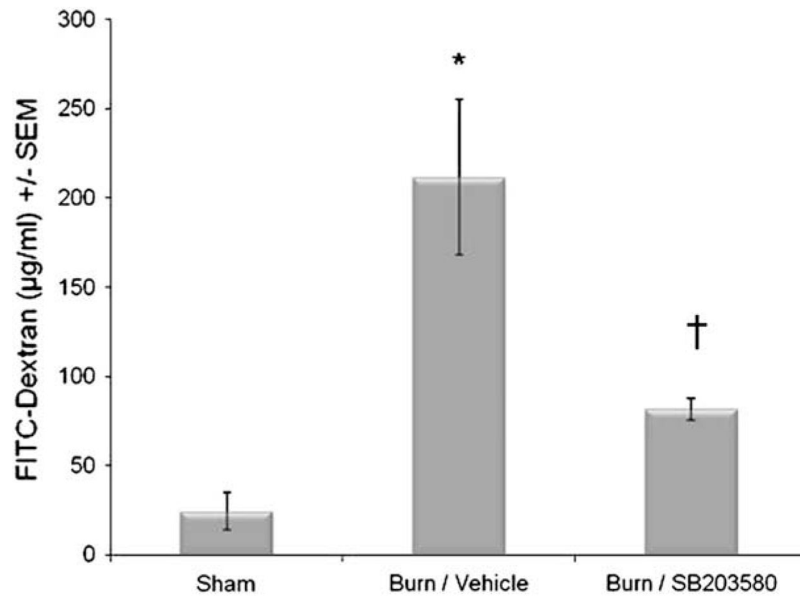

FIG 1.

Inhibition of p38 MAPK attenuates burn-induced intestinal permeability. Intestinal permeability (n 4 per group) was assessed using 4 kDa FITC-Dextran at 2 h following burn injury. There was a significant increase in intestinal permeability in burned animals injected with vehicle (DMSO). Intraperitoneal injection of the p38 MAPK inhibitor SB203580 (25 mg/kg) decreased burn-induced intestinal permeability. *$P < 0.03$ *versus* Sham, †$P < 0.05$ *versus* Burn/Vehicle.

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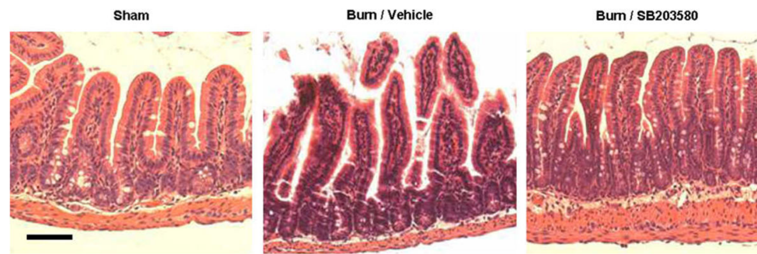

FIG 2.

Histologic evaluation of intestinal injury 2 h following burn. Segments of distal small bowel harvested 2 h following 30% TBSA steam burn (n 3 animals per group). Sections of distal ileum from sham animals show normal appearing villi. The distal ileum from animals 2 h following burn show early signs of histologic gut injury characterized by evidence of mucosal ulceration at the villous tips, and signs of early inflammatory cell infiltration. Sections from animals injected with the p38 MAPK inhibitor SB203580 have an appearance similar to sham, with normal appearing villi. Images viewed at ×20 magnification. Bar = 100 μm. (Color version of figure is available online.)

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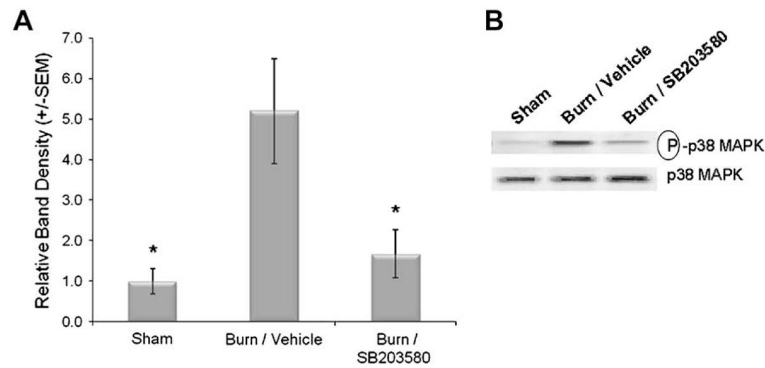

FIG 3.

Phosphorylation of intestinal p38MAPKfollowing severe burn. (A) Graph represents band densities of Western blots of distal ileum following burn (*n* 3 animals per group). There was a 5-fold increase in phosphorylated p38 MAPK in burned animals injected with vehicle (DMSO). Injection with the p38 MAPK inhibitor SB203580 (25 mg/kg) attenuated p38 MAPK phosphorylation to sham levels. (B) Representative Western blots of intestinal p38 MAPK and phosphorylated p38 MAPK following burn. *$P < 0.05$ *versus* Burn/Vehicle.

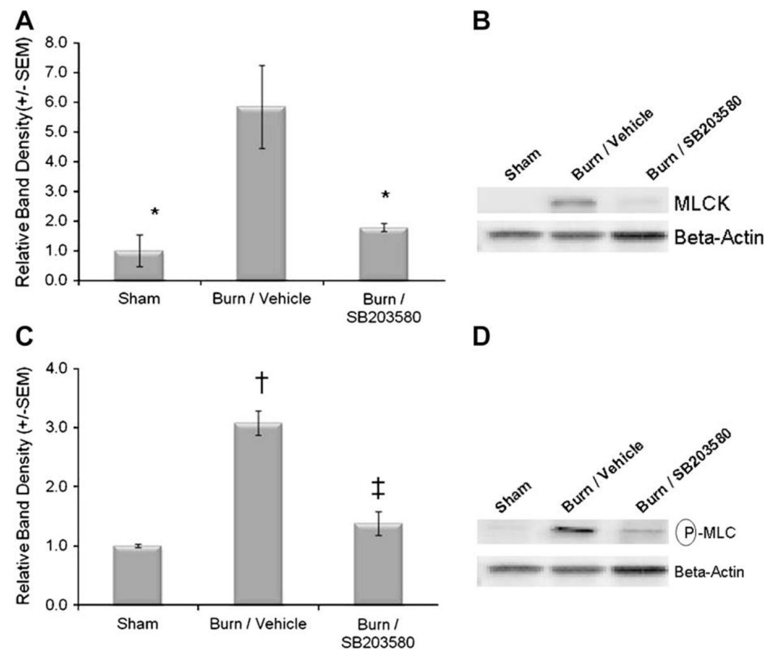

FIG 4.

Effect of p38 MAPK inhibition on burn-induced MLCK activation. (A) Graph representing intestinal MLCK protein expression 2 h following burn (*n* 3 animals per group). Intestinal MLCK expression increases 6-fold following burn. Intraperitoneal injection of SB203580 attenuates the burn-induced increase in MLCK. (B) Representative Western blots for intestinal MLCK. (C) Graph representing changes in phosphorylation of intestinal MLC. Increasing MLCK expression is associated with increased phosphorylation of MLC at 2 h following burn. Treatment with SB203580 attenuates the burn-induced increase in phosphorylated MLC. (D) Representative Western blots for intestinal phosphorylated MLC. β-Actin was used as a loading control. *$P < 0.03$ *versus* Burn/Vehicle, †$P < 0.001$ *versus* Sham, ‡$P < 0.01$ *versus* Burn/Vehicle.

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