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

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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.**

Key Words: **intestinal permeability; p38 MAPK; myosin light chain kinase; inflammation; intestine; burn; intestinal barrier; tight junction; gut.**

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

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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 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 ($n \geq 4$ 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 ($n \geq 3$ 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).

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.