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Calcium-Ask1-MKK7-JNK2-c-Src Signaling Cascade Mediates Disruption of Intestinal Epithelial Tight Junctions by Dextran Sulfate Sodium

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Abstract

Disruption of intestinal epithelial tight junctions is an important event in the pathogenesis of ulcerative colitis. Dextran sodium sulfate (DSS) induces colitis in mice with the symptoms similar to ulcerative colitis. However, the mechanism of DSS-induced colitis is unknown. We investigated the mechanism of DSS-induced disruption of intestinal epithelial tight junctions and barrier dysfunction in Caco-2 cell monolayers *in vitro* and mouse colon *in vivo*. DSS treatment resulted in disruption of tight junctions, adherens junctions and actin cytoskeleton leading to barrier dysfunction in Caco-2 cell monolayers. DSS induced a rapid activation of c-jun N-terminal kinase (JNK), and the inhibition or knockdown of JNK2 attenuated DSS-induced tight junction disruption and barrier dysfunction. In mice, DSS administration for 4 days caused redistribution of tight junction and adherens junction proteins from the epithelial junctions, which was blocked by JNK inhibitor. In Caco-2 cell monolayers, DSS increased intracellular Ca^{2+} concentration, and depletion of intracellular Ca^{2+} by BAPTA or thapsigargin attenuated DSS-induced JNK activation, tight junction disruption and barrier dysfunction. Knockdown of Ask1 or MKK7 blocked DSS-induced tight junction disruption and barrier dysfunction. DSS activated c-Src by a Ca^{2+} and JNK-dependent mechanism. Inhibition of Src kinase activity or knockdown of c-Src blocked DSS-induced tight junction disruption and barrier dysfunction. DSS increased Tyr-phosphorylation of occludin, ZO-1, E-cadherin and β -catenin. SP600125 abrogated DSS-induced Tyr-phosphorylation of junctional proteins. Recombinant JNK2 induced threonine phosphorylation and auto phosphorylation of c-Src. This study demonstrates that Ca^{2+} -Ask1-MKK7-JNK2-cSrc signaling cascade mediates DSS-induced tight junction disruption and barrier dysfunction.

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Statement of author contribution

Drs. Samak, Chaudhry and Gangwar were responsible for performing experiments, data analysis and organization, preparation of figures and editing manuscript. Dr. Narayan's contribution include conducting experiments and editing manuscript. Dr. Jaggar has contributed to this study by design of experiments and data interpretation. Dr. Rao is responsible for the design of experiments, interpretation of data and manuscript writing.

Keywords

barrier function; epithelium; occludin; ZO-1; actin; stress; phosphorylation

INTRODUCTION

A major function of the gastrointestinal epithelium is to form a physical barrier between the hostile environment of the gastrointestinal lumen and the sub-epithelial tissue. The epithelial tight junctions provide this barrier against the paracellular penetration of noxious substances from the gut lumen. Immediately basal to tight junction is the adherens junction, which is known to indirectly regulate the integrity of tight junctions [1]. These intercellular junctions associate with the perijunctional actin cytoskeleton and numerous signaling molecules to form an integrated functional unit called the apical junctional complex (AJC)[2]. The integrity of AJC is compromised in numerous gastrointestinal diseases, including inflammatory bowel diseases such as Crohn's disease and ulcerative colitis [3, 4]. Colitis induced by dextran sodium sulfate (DSS) is an extensively applied animal model of the human ulcerative colitis [5, 6]. This model exhibits clinical features very similar to those in ulcerative colitis patients. However, the mechanism involved in DSS-induced colitis is poorly understood. Similar to ulcerative colitis patients, DSS-induced colitis in rodents is associated with increased intestinal mucosal permeability [7–10]. Elevated mucosal permeability appears to precede the onset of inflammatory process [8, 9]. Although epithelial barrier dysfunction in DSS-induced colitis is well documented, the precise mechanism involved in DSS-induced epithelial barrier dysfunction is poorly understood. DSS-induced colitis was found to be associated with a depletion of zona occludens-1 (ZO-1), a tight junction protein, in the intestinal epithelium, suggesting that tight junctions may be altered [11].

Tight junction assembly involves at least four types of transmembrane proteins: occludin, tricellulin, claudins, and junctional adhesion molecules [12]. Transmembrane proteins interact with cytoplasmic adapter proteins such as ZO-1, which anchors the transmembrane proteins to the actin cytoskeleton [13]. A significant body of evidence indicates that a variety of intracellular signaling molecules are associated with AJC and that activities of such signaling molecules regulate the integrity of epithelial tight junctions [14]. The major groups of signaling elements that regulate tight junctions include protein kinases and protein phosphatases, such as Src kinases [15, 16], protein kinase C [17–19], MAP kinases [20–22], protein tyrosine phosphatase [14, 23, 24] and protein serine/threonine phosphatases [25, 26].

c-Jun N-terminal kinases (JNKs), a subgroup of MAP kinases, are activated by various types of stress, such as UV irradiation, osmotic stress, heat shock, as well as by direct activation of cytokine and T toll-like receptors [27]. JNK activation causes tissue injury by induction of apoptosis, production of pro-inflammatory cytokines and delayed differentiation[28]. Three JNK isoforms are JNK1, JNK2, and JNK3. While JNK3 expression is limited to neural cells, JNK1 and JNK2 are ubiquitously distributed in different cell types [28]. Although JNK activation was detected in inflamed colonic mucosa of patients with inflammatory bowel disease [29], the role of JNK in the disease activity is unclear. JNK inhibitor SP600125

ameliorated trinitrobenzoyl sulfonic acid-induced colitis in mice, predominantly by inhibiting JNK activity in the infiltrating inflammatory cells [30].

In the present study, we investigated the direct effect of DSS on the epithelial integrity and determined the role of JNK in DSS-induced tight junction disruption and barrier dysfunction using Caco-2 cell monolayers and mouse intestine. We further investigated the importance of intracellular calcium ($[Ca^{2+}]_i$) in DSS-induced JNK2 activation, the JNK-mediated c-Src activation and c-Src-mediated Tyr phosphorylation of tight junction and adherens junction proteins.

METHODS AND MATERIALS

Chemicals

Cell culture supplies, transfection reagents, Fura-2AM, pluronic acid and phalloidin (Cy3 or AlexaFluor-488 conjugated) were procured from Cellgrow® (Manassas, VA) or Invitrogen (Carlsbad, CA). Transwells were purchased from Costar (Cambridge, MA). SP600125, thapsigargin and PP2 were from EMD Chemicals (San Diego, CA). Other chemicals were purchased from either Sigma Aldrich (St. Louis, MO) or Thermo Fisher Scientific (Tustin, CA). JNK2 and c-Src recombinant proteins were purchased from MyBioSource (San Diego, CA).

Antibodies

Anti-JNK(pTpY^{183/185}), anti-c-Src(pY⁴¹⁸), anti-phospho-threonine, anti-ZO-1, anti-occludin, and anti-claudin-4 antibodies were purchased from Invitrogen (Carlsbad, CA). Anti-JNK, anti-Ask1, anti-MKK7 and anti-c-Src antibodies were purchased from Millipore (Billerica, MA). HRP-conjugated anti-mouse IgG, HRP-conjugated anti-rabbit IgG and anti- β -actin antibodies were obtained from Sigma Aldrich. AlexaFluor-488-conjugated anti-mouse IgG and Cy3-conjugated anti-rabbit IgG were purchased from Molecular Probes (Eugene, OR). Anti-E-cadherin, anti- β -catenin, and biotin-conjugated anti-p-Tyr antibodies were purchased from BD Biosciences (Billerica, MA). FITC-conjugated anti-mouse Gr-1 antibodies were from MyBioSource (San Diego, CA).

Antisense oligonucleotides and siRNA

Antisense oligonucleotides for JNK1 (AS-Jnk1) and JNK2 (AS-Jnk2) and the scrambled sequence for AS-Jnk1 (MS-Oligo) were prepared as described previously [31]. Human c-Src-specific siRNA and corresponding control RNA were purchased from Dharmacon (Lafayette, CO) and OriGene (Rockville, MD). Sets of siRNA against Ask1 and MKK7 were purchased also from OriGene.

Cell culture and transfection

Caco-2 cells (ATCC, Rockville, MD) were grown under standard cell culture conditions as described before [32]. Experiments were conducted using cells grown in transwell inserts of varying diameters (6.5–24 mm) for 10–15 days.

Cells grown in 6-well costar plates for 24 h showing $\approx 75\%$ confluence were transfected using serum-free Opti-MEM[®], 150 nM oligonucleotides (MS-oligo, AS-Jnk1, AS-Jnk2, c-Src siRNA, siAsk1, siMKK7 and nonspecific RNA) and 3.15 μ l of Oligofectamine[®] as described previously [31]. Transfected cells were seeded onto transwell inserts. Experiments were performed on day 3 or 4 after seeding.

Cell treatments

Cell monolayers in transwell inserts were incubated with 2.5–3.0% DSS in DMEM (apically). SP600125 (1 μ M) was administered 50 min prior to DSS treatment and PP2 (10 μ M) administered 30 min prior to DSS. For Ca^{2+} depletion, DSS was prepared in DMEM lacking Ca^{2+} . Depletion of $[\text{Ca}^{2+}]_i$ was achieved by incubation of cells with 10 μ M BAPTA-AM or 1 μ M thapsigargin for 30 min.

Epithelial barrier function

Transepithelial electrical resistance (TER) was measured using a Millicell-ERS Electrical Resistance System and macromolecular permeability evaluated by measuring unidirectional flux of FITC-inulin as described previously [31]. The basal TER values for Caco-2 cell monolayers in these experiments were 400–500 Ohms/cm².

Cell viability assay

Lactate dehydrogenase release from cells into medium and the mitochondrial dehydrogenase activity (WST assay) in cells were measured using commercial kits as described before to evaluate cell viability [31].

DSS administration in mice

All animal experiments were performed according to the protocols approved by the institutional animal care and use committee at the University of Tennessee Health Science Center, Memphis. Adult female mice (C57BL/6; 12–14 weeks) were used for these studies. Colitis was induced by administering 2.5% (w/v) DSS in drinking water for 4 or 6 days. Body weights, spleen and colon length and weights were measured. Stool consistency and rectal bleeding were graded on 0–3 scales. Stool was graded as: 0 - well-formed pellets, 1 - semisolid stools that do not adhere to anus, 2 - semisolid stool that adhere to anus, and 3 - liquid stool that adhere to anus. Bleeding was graded as: 0 – no blood in stool as tested by hemocult analysis (ColoScreen-ES, Helena), 1 – positive hemocult, 2 – positive hemocult with trace of blood in stool, and 3 – visible rectal bleeding.

Fluorescence microscopy

Caco-2 cell monolayers and Cryosections (10 μ m thickness) of colon were fixed in 3% paraformaldehyde for 15 min at room temperature. Following permeabilization with 0.2% Triton X-100[™], sections were blocked and stained for different proteins as described before [31]. The fluorescence was examined by using a Zeiss LSM 510/710 laser scanning confocal microscope and 20 $\times$ objective lens. Images from x-y (1 μ m) sections were collected using LSM Pascal or Zen software. Images from sections were stacked using Image J (NIH) and processed by Adobe Photoshop (Adobe Systems, San Jose, CA).

Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured as previously described[33]. Briefly, serum-starved Caco-2 cell monolayers on glass-bottom microwell dishes (MatTek; Ashland, MA) were incubated with Fura-2AM (10 μ M) in 0.5% pluronic acid for 30 min. Fura-2-loaded cells were alternately excited at 340 or 380 nm using a PC-driven hyper-switch (Ionoptix; MA, USA). Ratios were collected every second at 510 nm using a Dage MTI iCCD camera and Ionwizard software (Ionoptix). $[Ca^{2+}]_i$ was calculated using the following equation: $[Ca^{2+}]_i = K_d (R - R_{min}) (S_{f2}) / (R_{max} - R) (S_{b2})$, where R is the 340/380 nm ratio, R_{min} and R_{max} are the minimum and maximum ratios determined in Ca^{2+} -free and saturated Ca^{2+} solutions, respectively, S_{f2}/S_{b2} is the Ca^{2+} free/ Ca^{2+} -replete ratio of emissions at 380 nm excitation, and K_d is the dissociation constant for Fura-2. R_{min} , R_{max} , S_{f2} , and S_{b2} were determined by increasing the Ca^{2+} permeability of Caco-2 cells with ionomycin (10 μ M), and perfusing cells with a high- Ca^{2+} (10 mM) or Ca^{2+} -free (10 mM EGTA) solution. The in situ apparent dissociation constant (K_d) for Fura-2 used in this study was 224 nM. Eight to ten cells in each monolayer were analyzed simultaneously, and the experiments were repeated in 3–5 monolayers.

c-Src activation by JNK2 *in vitro*

Recombinant c-Src (500 ng) was incubated with or without recombinant JNK2 (200 ng) in the presence or absence of ATP (0.5 mM) and in the presence or absence of 1 μ M SP600125 or 10 μ M PP2 in Tris kinase buffer (50 mM Tris-HCl, pH 7.5, 150 mM sodium chloride, 0.5 mM ethylene diaminetetraacetic acid, 0.02% Triton X-100, and 2 mM DTT) for one hour at 30°C. Samples were then immunoblotted for p-Thr, c-Src(pY418), c-Src or JNK.

Immunoprecipitation

Caco-2 cell monolayers were washed with phosphate buffered saline and proteins extracted in heated lysis buffer-D (0.3% sodium dodecyl sulfate in 10 mM Tris buffer, pH 7.4, containing 1 mM sodium orthovanadate, 10 mM sodium fluoride, 1 mM phenyl methyl sulfonylfluoride, and 10 μ l/ml protease inhibitors cocktail). Phospho-Tyr was immunoprecipitated as described before using biotin-conjugated anti-phospho-Tyr antibody [32]. Immunoprecipitates were immunoblotted for AJC proteins. Similarly, extracts from colonic mucosa was used to immunoprecipitate phosphor-Tyr and immunoblotted for ZO-1.

Immunoblot analysis

Proteins in cell extracts were separated by sodium dodecyl sulfate-polyacrylamide gel (7%) electrophoresis and transferred to polyvinylidene fluoride membranes. Membranes were immunoblotted for different proteins as described before [31].

Statistical analyses

Comparison between two groups was made by the Student's t tests (unpaired) for grouped data. The significance in all tests was derived at the 95% or greater confidence level.

RESULTS

DSS disrupts AJC and induces barrier dysfunction in Caco-2 cell monolayers

Evidence suggests that DSS-induced colitis in mice may involve elevated mucosal permeability [5, 7, 8, 11]. But, the role of tight junction disruption in DSS-induced mucosal permeability and whether DSS directly affects the epithelial cells are not well understood. Therefore, studies were conducted to evaluate the effect of DSS on epithelial junctional complexes and barrier function in Caco-2 cell monolayers. Treatment of cell monolayers with DSS for 3 hours dose-dependently reduced TER (Fig. 1A) and increased transepithelial inulin permeability (Fig. 1B). DSS treatment did not affect cell viability as assessed by lactate dehydrogenase release into medium and cellular mitochondrial activity (Fig. 1C). Stain for actin cytoskeleton was reorganized by DSS treatment (Fig. 1D). DSS reduced F-actin stain at the upper mid region of the epithelial cell by 30 min, whereas actin organization at the apical and basal regions was unaffected until 60 min. DSS treatment for 2 hours caused a redistribution of tight junction proteins, occludin and ZO-1 from the intercellular junctions into the intracellular compartment (Fig. 1E). Junctional distribution of E-cadherin and β -catenin was also reduced by DSS treatment (Fig. 1F).

JNK activity mediates DSS-induced tight junction disruption and barrier dysfunction

DSS treatment caused a rapid increase in the levels of p-JNK1/2 (JNK1/2^{pT183/pY185}) in Caco-2 cells without considerable effect on the total JNK1/2 levels (Fig. 2A). Pretreatment of cell monolayers with SP600125 (a JNK inhibitor) significantly attenuated DSS-induced decrease in TER (Fig. 2B), increase in inulin permeability (Fig. 2C). SP600125, by itself had no significant effect on TER or inulin permeability. SP600125 also attenuated DSS-induced redistribution of occludin and ZO-1 from the intercellular junctions (Fig. 2D). Transfection of Caco-2 cells with AS-Jnk2, the JNK2-specific antisense oligonucleotide [31], reduced JNK2 levels (Fig. 2E) and significantly attenuated DSS-induced decrease in TER (Fig. 2F), increase in inulin permeability (Fig. 2G) and redistribution of occludin and ZO-1 (Fig. 2H).

DSS administration disrupts AJC in mouse colon *in vivo* by a JNK-dependent mechanism

To determine the influence of DSS on colonic mucosal barrier function *in vivo* mice were treated with 2.5% DSS (w/v) in drinking water for 4 days and evaluated its effect on the integrity of epithelial tight junction and adherens junction in colon. DSS administration resulted in significant loss of body weight (Fig. 3A). Administration of SP600125 by itself reduced the body weight, but DSS administration did not further alter the body weight in the presence of SP600125. Stool consistency was only slightly affected, which was significantly attenuated by SP600125 administration (Fig. 3B). DSS did not cause rectal bleeding at this stage, but slight hemoccult was detected in DSS-treated mice (Fig. 3B). Length and weight of colon were unaffected by DSS or SP600125 (Fig. 3C–D). Similarly, spleen weight and length were also unaffected by DSS or SP600125 (data not shown).

DSS administration for 4 days did not show any sign of colitis (Fig. 4A) nor altered the gross morphology of mucosa and sign of inflammation (Fig. 4B). On the other hand, DSS treatment for 6 days did result in colitis. Inflammatory process was further assessed by co-staining cryosections of colon for Gr1, a marker of polymorphonuclear neutrophil (PMN)

and F-actin. DSS administration for 4 days did not induce neutrophil infiltration, but 6 days of DSS administration resulted in significant infiltration of PMN in colonic mucosa (Fig. 4C). DSS treatment for 4 days increased p-JNK levels in colonic mucosa (Fig. 4D) and caused depletion of occludin and ZO-1 at the epithelial junctions (Fig. 5). SP600125 administration attenuated this DSS-induced depletion of tight junction proteins at the intercellular junctions. DSS treatment also reduced junctional organization of E-cadherin and β -catenin in distal colon, which was blocked by SP600125 administration (Fig. 6).

Calcium signaling mediates DSS-induced JNK activation and tight junction disruption in Caco-2 cell monolayers

Studies were conducted to investigate the upstream mechanism involved in DSS-induced JNK activation. A recent study indicated a potential role of $[Ca^{2+}]_i$ in JNK activation and regulation of epithelial tight junctions during osmotic stress [33]. Therefore, we examined the effect of DSS on $[Ca^{2+}]_i$ in Caco-2 cells. DSS rapidly increased $[Ca^{2+}]_i$, and this effect was partially attenuated by either removal of extracellular Ca^{2+} or depletion of endoplasmic reticulum (ER) Ca^{2+} using thapsigargin, an ER Ca^{2+} -ATPase inhibitor (Fig. 7A & B). Simultaneous treatment with thapsigargin and extracellular Ca^{2+} depletion completely blocked DSS-induced rise in $[Ca^{2+}]_i$ (Fig. 7B). Consistent with this observation, extracellular Ca^{2+} depletion or thapsigargin partially reduced DSS-induced JNK activation (Fig. 7C). Treatment of cells with 1,2-bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra acetic acid tetra(acetoxymethyl) ester (BAPTA-AM) abolished DSS-induced JNK activation (Fig. 7C). Pretreatment of cell monolayers with BAPTA-AM, thapsigargin or extracellular Ca^{2+} depletion attenuated DSS-induced decrease in TER (Fig. 7D), increase in inulin permeability (Fig. 7E), and redistribution of occludin and ZO-1 from intercellular junctions (Fig. 7F). BAPTA-AM, thapsigargin or extracellular calcium depletion by itself had no significant effect on JNK activation, TER or inulin permeability.

Ask1 and MKK7 mediates DSS-induced tight junction disruption in Caco-2 cell monolayers

To establish the link between $[Ca^{2+}]_i$ and JNK2 activation we evaluated the effects of specific knockdown of Ask1 or MKK7, the signaling elements known to play role in activation of JNK2. Specific siRNAs targeting 3 different sequences of Ask1 and MKK7 genes were transfected to Caco-2 cells, which significantly reduced the levels of corresponding protein (Fig. 8A–C). In NS-RNA-transfected cell monolayers, DSS treatment significantly reduced TER in a time-dependent manner (Fig. 8D). Knockdown of Ask1 or MKK7 significantly attenuated DSS-induced decline in TER. Ask1 or MKK7 knockdown also significantly blocked DSS-induced inulin permeability (Fig. 8E). DSS induced redistribution of occludin and ZO-1 from the intercellular junctions of NS-RNA-transfected cell monolayers (Fig. 8F). Knockdown of either Ask1 or MKK7 preserved the junctional organization of occludin and ZO-1 during DSS treatment.

Calcium and JNK-dependent activation of c-Src mediates DSS-induced tight junction disruption

Studies were targeted to investigate the potential signaling down stream to JNK activation during DSS-induced tight junction disruption. Previous studies have demonstrated that c-Src

activation leads to epithelial tight junction disruption [15]. Therefore, in the present study, we investigated the role of c-Src in DSS-induced tight junction disruption. Incubation of Caco-2 cell monolayers with DSS for 30 min increased the level of c-Src(pY⁴¹⁸), which was blocked by SP600125, PP2 (Src kinase inhibitor), thapsigargin, and extracellular Ca²⁺ depletion (Fig. 9A). The DSS-induced increase in pJNK1/2, on the other hand, was unaffected by PP2 (Fig. 9B). PP2 treatment significantly blunted DSS-induced decrease in TER (Fig. 9C) and increase in inulin permeability (Fig. 9D). PP2 also attenuated DSS-induced redistribution of occludin and ZO-1 from the intercellular junctions (Fig. 9E). Transfection of Caco-2 cells with c-Src-specific siRNA significantly reduced the level of c-Src (Fig. 9F) and attenuated DSS-induced decrease in TER (Fig. 9G) and increase in inulin permeability (Fig. 9H). Knockdown of c-Src also attenuated DSS-induced redistribution of occludin and ZO-1 from the intercellular junctions (Fig. 9I). The role of c-Src in DSS-induced barrier dysfunction and tight junction disruption was further confirmed by specific knockdown by using another set of siRNA. The data, similar to the effect of above siRNA, are presented in the supplemental figure 1.

DSS-induced barrier dysfunction is associated with JNK-dependent Tyr-phosphorylation of AJC proteins

Previous studies demonstrated that hydrogen peroxide-induced disruption of epithelial tight junctions is associated with c-Src-mediated phosphorylation of occludin [15], and that occludin phosphorylation on specific Tyr residues results in a loss of its interaction with ZO-1, leading to disruption of tight junctions [16]. The involvement of c-Src activity in the present study raised the question whether Tyr-phosphorylation of junctional proteins is associated with DSS-induced tight junction disruption. Therefore, we investigated the influence of DSS on Tyr-phosphorylation of tight junction and adherens junction proteins. Incubation of Caco-2 cell monolayers with DSS for 1 h induced a robust increase in Tyr-phosphorylation of occludin, ZO-1, E-cadherin and β -catenin (Fig. 10A). Pretreatment of cell monolayers with SP600125 abrogated DSS-induced Tyr-phosphorylation of these proteins. Claudin-4 was found to be Tyr-phosphorylated in control cell monolayers, which was unaffected by DSS or SP600125 (Fig. 10A). The total amounts of AJC proteins were unaffected by DSS or SP600125 (Fig. 10B). Immunoprecipitation of phospho-Tyr and immunoblot analysis showed that DSS administration for 4 days induced a dramatic increase and Tyr-phosphorylation of ZO-1 in mouse colonic mucosa, which was blocked by SP600125 administration (Fig. 10C).

JNK2 directly phosphorylates c-Src and activates its auto phosphorylation

Above studies show that DSS activates c-Src by a JNK-dependent mechanism. The mechanism involved in this JNK2 activity is unclear. This effect of JNK2 may be caused by a direct interaction with c-Src or an indirect mechanism involving other proteins that are known to regulate c-Src activity. To determine whether or not JNK2 directly activates c-Src we incubated recombinant c-Src with recombinant JNK2 in the presence or absence of ATP and with or without selective kinase inhibitors. Results show that incubation with JNK2 and ATP increased the auto phosphorylation of c-Src on Y418, an indicator of activation of c-Src (Fig. 11). In the absence of JNK2, incubation with ATP slightly elevated auto phosphorylation of c-Src. JNK2-mediated activation of c-Src was blocked by SP600125

(JNK inhibitor) as well as PP2 (Src inhibitor). Activation of c-Src by JNK2 was accompanied by the phosphorylation of c-Src on threonine residue(s).

DISCUSSION

Ulcerative colitis is a chronic inflammatory disease of the colon and rectum of multifactorial etiology [3]. DSS-induced colitis in mice is a well-established model of ulcerative colitis [6]. DSS administration induces erosion and ulceration in the colonic mucosa that is associated with infiltration of inflammatory cells. These symptoms of DSS-induced colitis are similar to those seen in ulcerative colitis patients. However, the mechanisms associated with DSS-induced colitis are poorly understood. Disruption of epithelial barrier and increased mucosal permeability are associated with DSS-induced colitis, and few reports suggest that barrier dysfunction may occur prior to the onset of inflammatory process. Here we provide evidence that a rapid activation of JNK2 is involved in DSS-induced disruption of the AJC in Caco-2 cell monolayers *in vitro* and in mouse colonic epithelium *in vivo* at an initial stage prior to inflammation. Furthermore, this study shows that Ca^{2+} -Ask1-MKK7-JNK2-cSrc signaling cascade mediates DSS-induced tight junction disruption and barrier dysfunction.

Evidence indicates that DSS-induced colitis in mice is associated with colonic mucosal permeability [5, 7, 8, 11]. However, it is not clear whether the increased permeability was caused by a direct effect on the epithelial junctions. A previous study showed that DSS decreases TER in Caco-2 cell monolayers [34], but it was unclear whether the decline in TER was caused by changes in the transcellular or paracellular transports. The observation in the present study that DSS decreases TER and increases inulin permeability in Caco-2 cell monolayers without altering cell viability indicates that DSS can directly affect the epithelial barrier function. Redistribution of tight junction and adherens junction proteins from the intercellular junctions demonstrates that DSS disrupts both tight junctions and adherens junctions in the intestinal epithelium. DSS-induced disruption of tight junction and adherens junction was associated with the reorganization of actin cytoskeleton. Actin reorganization occurred predominantly in the upper mid-region of epithelial cells, indicating that DSS impacts the adherens belt region of the actin cytoskeleton that interacts with the tight junction and adherens junction protein complexes.

A rapid increase in pJNK in DSS-treated cells indicates that DSS activates JNK1 and JNK2 in Caco-2 cell monolayers. A significant attenuation of DSS-induced barrier dysfunction and redistribution of occludin and ZO-1 from the junctions by SP600125 demonstrate that JNK activity mediates DSS-induced tight junction disruption in Caco-2 cell monolayers. Although SP600125 is a well-established JNK inhibitor and extensively used to probe the cellular of JNK activity, it inhibits both JNK1 and JNK2 activities. Attenuation of DSS-induced barrier dysfunction and redistribution of occludin and ZO-1 by specific knockdown of JNK2 indicates the importance of JNK2 isoform in the DSS-induced tight junction disruption. The antisense oligo used in this study was previously shown to specifically knockdown JNK2, without altering the levels of JNK1. Selective activation of JNK1 by EGF failed to disrupt tight junctions in Caco-2 cell monolayers, JNK1 activation rather reduced inulin permeability.

The role of JNK activity in DSS-induced barrier dysfunction was further confirmed in mouse colon *in vivo*. DSS administration for 4 days resulted in redistribution of occludin, ZO-1, E-cadherin and β -catenin from the junctions in colonic epithelium, indicating the DSS-induced loss of tight junction and adherens junction integrity. Attenuation of DSS-induced redistribution of tight junction and adherens junction proteins by SP600125 administration demonstrates the role of JNK activity in DSS-induced tight junction and adherens junction disruption in mouse colon *in vivo*. DSS administration for 4 days resulted in low-grade diarrhea, which was prevented by SP600125 administration. Histopathology and staining colonic cryosections for PMN marker, Gr1, indicated that inflammatory process was absent in colon at 4 days of DSS treatment. On the other hand, colon from mice at 6 days of DSS treatment showed significant PMN infiltration. Disruption of tight junctions and adherens junctions at 4 days of DSS treatment indicates that tight junction disruption and barrier dysfunction occur prior to the onset of inflammatory process, which is in agreement with the previous suggestions that mucosal permeability may precede the inflammatory process [8, 9]. These *in vivo* studies establish the physiologic relevance of the observations made in Caco-2 cell monolayers.

Previous study showed that the JNK inhibitor, SP600125, partially reduced DSS-induced wasting and colonic mucosal ulceration in mice [30], whereas another study indicated the lack of a role for JNK in IBD pathogenesis [35]. Therefore, the precise role of JNK activation in the pathogenesis of colitis is unknown. Interestingly, our study shows that DSS-induced diarrhea and disruption of tight junctions and adherens junctions were attenuated by SP600125 administration. Therefore, JNK-mediated disruption of AJC may play an important role in DSS-induced colitis. Specific inhibition of JNK or its up stream or down stream signals may serve as therapeutic target in the treatment of ulcerative colitis.

To determine the upstream signal involved in DSS-induced JNK activation we investigated the potential role of $[Ca^{2+}]_i$ in DSS-induced JNK activation and barrier dysfunction. Ca^{2+} signaling is known to play roles in both assembly and disruption of tight junctions [33]. In the present study, we found that DSS rapidly increases $[Ca^{2+}]_i$, which can be partially reduced by either extracellular Ca^{2+} depletion or blocking intracellular ER Ca^{2+} release by thapsigargin. A combined absence of extracellular Ca^{2+} and ER Ca^{2+} release abrogated DSS-induced rise in $[Ca^{2+}]_i$, indicating that DSS increases $[Ca^{2+}]_i$ by stimulating both extracellular Ca^{2+} influx and ER Ca^{2+} release. Blocking the $[Ca^{2+}]_i$ elevation with BAPTA-AM, thapsigargin or extracellular Ca^{2+} depletion attenuated DSS-induced increase in pJNK levels, indicating that DSS-induced JNK activation is mediated by $[Ca^{2+}]_i$. Blocking $[Ca^{2+}]_i$ significantly dampened DSS-induced barrier dysfunction and redistribution of tight junction proteins from the intercellular junctions. These results demonstrate that rise in $[Ca^{2+}]_i$ is essential for DSS-induced tight junction disruption.

JNK activation by a calcium-dependent mechanism raised the question, what is the mechanism involved in calcium-induced JNK activation. There is no evidence in the literature supporting a direct activation of JNK by calcium. However, the known upstream regulator of JNK2 activation is MKK7 [36]. Therefore, we investigated the role of MKK7 in DSS-induced tight junction disruption. Knockdown of MKK7 in Caco-2 cell monolayers significantly blocked DSS-induced decline in TER and increase in inulin permeability,

indicating the requirement of MKK7 in the mechanism of DSS-induced barrier dysfunction. MKK7 depletion also attenuated DSS-induced loss of junctional organization of tight junction proteins, occludin and ZO-1. These data demonstrate the role of MKK7. Upstream to MKK7 is Ask1, a signaling molecule known to be regulated by calcium via calmodulin kinase [36]. Therefore, we investigated the potential role of Ask1 in DSS-induced tight junction disruption. Knockdown of Ask1 significantly blocked DSS-induced drop in TER, rise in inulin permeability and loss of junctional organization of occluding and ZO-1, indicating that Ask1 is an important component of mechanism involved in DSS-induced tight junction disruption. These data demonstrate that Ask1 and MKK7 mediate calcium-induced JNK2 activation. This is the first report of tight junction regulation by Ask1 and MKK7.

We further investigated the signal downstream to JNK activation in DSS-induced tight junction disruption and barrier dysfunction. Previous studies demonstrated that c-Src activation leads to disruption of epithelial tight junctions and barrier dysfunction, and such a mechanism is involved in hydrogen peroxide-induced tight junction disruption in Caco-2 cell monolayers [15]. The mechanism of c-Src-mediated tight junction disruption involves Tyr-phosphorylation of tight junction and adherens junction proteins [16, 37]. Tyr-phosphorylation of tight junction and adherens junction proteins are known to weaken the integrity of AJC [38, 39]. Therefore, we investigated the potential role of c-Src activation in DSS-induced tight junction disruption. DSS rapidly increased the level of c-Src(pY418), indicating that DSS activates c-Src. DSS-induced c-Src activation was prevented by BAPTA-AM, thapsigargin and extracellular Ca^{2+} depletion as well as by SP600125, indicating that DSS activates c-Src by a Ca^{2+} and JNK-dependent mechanism. Inhibition of Src kinase activity or knockdown of c-Src attenuated DSS-induced tight junction disruption and barrier dysfunction. Therefore, c-Src activation is an important event downstream to JNK activation during DSS-induced tight junction disruption.

DSS-induced c-Src activation and tight junction disruption was associated with Tyr phosphorylation of occludin, ZO-1, E-cadherin, and β -catenin in Caco-2 cell monolayers. Prevention of DSS-induced Tyr-phosphorylation of tight junction and adherens junction proteins by SP600125 indicated that JNK activity mediates DSS-induced Tyr-phosphorylation, likely via c-Src activation. DSS administration in mice also caused Tyr-phosphorylation of ZO-1 in colonic mucosa, which was prevented by SP600125. Therefore, the mechanism involving JNK-mediated Tyr-phosphorylation of tight junction proteins, likely via c-Src activation, also exists in the mouse colonic epithelium *in vivo*. Phosphorylation of occludin on Y398 and Y402 was previously shown to attenuate its interaction with ZO-1 and weaken tight junction integrity [38, 39]. It is likely that DSS induces Tyr-phosphorylation of occludin on Y398 and Y402 residues.

The role of JNK activity in c-Src activation in DSS-treated cells raised question whether the effect is caused by a direct interaction of JNK2 with c-Src or it is indirect mediated by other proteins. At this time, there is no report to support a direct interaction between JNK2 and c-Src. To determine a potential direct interaction we conducted *in vitro* experiments using recombinant JNK2 and c-Src proteins. Incubation of c-Src with JNK2 and ATP induced a robust increase in the level of c-Src(pY418). Auto phosphorylation of c-Src on Y418 is an

indicator of c-Src activation [40]. It is unclear at this time how JNK2 activates c-Src. Our data show that increase in c-Src auto phosphorylation by JNK2 is associated with threonine phosphorylation of c-Src, and therefore, needs further investigations to determine the precise mechanism of JNK2-induced activation of c-Src.

These studies in Caco-2 cell monolayers *in vitro* and mouse colon *in vivo* therefore demonstrate that DSS stimulates extracellular Ca^{2+} influx and ER Ca^{2+} release to elevate the $[\text{Ca}^{2+}]_i$ (Fig. 12). $[\text{Ca}^{2+}]_i$ activates JNK2 likely by Ask1 and MKK7-mediated mechanism, and JNK2 activates c-Src likely by direct phosphorylation of c-Src on threonine residues. Src activation leads to Tyr-phosphorylation of occludin, likely on Y398 and Y402 residues, resulting in weakening of tight junctions and causing barrier dysfunction. Tyrosine phosphorylation of ZO-1 and adherens junction proteins may also contribute to disruption of tight junction disruption. Disruption of tight junctions and barrier dysfunction may lead to mucosal translocation of luminal toxins and pathogens to induce inflammatory process and colitis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Abbreviations

AJC	apical junctional complex
As-Jnk1/2	antisense oligonucleotides for JNK1 and JNK2
Ask1	apoptosis BAPTA-AM, 1,2-bis(<i>o</i> -aminophenoxy)ethane-N,N,N'-tetraacetic acid tetra(acetoxymethyl) ester
CFM	calcium free medium
DSS	dextran sodium sulfate
ER	endoplasmic reticulum
FITC	fluorescein isothiocyanate
MKK7	MAP kinase kinase
MS-oligo	missense oligonucleotide
NS-RNA	non specific RNA
pJNK1/2	JNK1/2(pT ¹⁸³ pY ¹⁸⁵)
PMN	polymorphonuclear neutrophil
p-cSrc	c-Src(pY ⁴¹⁸)
PP2	4-amino-5-(4-chlorophenyl)-7-(<i>t</i> -butyl)pyrazolo[3,4- <i>d</i>]pyrimidine

SP600125	(anthra[1,9-cd]pyrazol-6(2H)-one)
TER	transepithelial electrical resistance
TG	thapsigargin
ZO-1	zona occludens-1

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Figure 1. DSS disrupts apical junctional complexes in Caco-2 cell monolayers

A–C: Caco-2 cell monolayers were incubated with DSS at varying concentrations for 3 hours. TER (**A**) and inulin permeability (**B**) were measured. Cell viability evaluated after 4 hour DSS treatment by measuring LDH activity in incubation medium and cellular mitochondrial activity by WST assay (**C**). Values are mean $\pm$ SEM ($n = 6$). Asterisks indicate the values that are significantly ($P < 0.05$) different from corresponding control (0% DSS) values. **D–F:** Cell monolayers incubated with or without DSS for 30–90 min were fixed and stained for F-actin (**D**), tight junction proteins (**E**) and adherens junction proteins

(F) by immunofluorescence method. For actin cytoskeleton, 2 μ m stacked optical sections at the apical, upper middle and distal regions of cell monolayer are presented.

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Figure 2. JNK activity mediates DSS-induced tight junction disruption in Caco-2 cell monolayers

A: Caco-2 cell monolayers were incubated with DSS (2.5%) for varying times and cell extracts were immunoblotted for p-JNK, JNK and actin. **B–D:** Cell monolayers were incubated with (■, □) or without (●, ○) SP600125 (1 μM) 30 min prior to incubation with (●, ■) or without (○, □) 2% DSS for varying times. TER (**B**) and inulin permeability (**C**) were measured at varying times. Values are mean ± SEM ($n = 6$). Asterisks indicate the values that are significantly ($P < 0.05$) different from corresponding control values (○), and # symbol indicates values that are significantly different from corresponding values for DSS group (●). Cell monolayers were fixed and stained for tight junction proteins. Fluorescence images were collected by confocal microscopy (**D**). **E–H:** Caco-2 cell monolayers were transfected with missense (MS-Oligo; ●, ○) or antisense (As-Jnk1 or As-Jnk2; ■, □) oligonucleotides. Protein extracts were immunoblotted for JNK2 and band densities quantitated (**E**). Transfected cell monolayers were incubated with (●, ■) or without (○, □) 2% DSS for varying times. TER (**F**) and inulin permeability (**G**) were measured at varying times. Values are mean ± SEM ($n = 6$). Asterisks indicate values that are significantly ($P < 0.05$) different from corresponding control values. After 3 hours of DSS treatment, cell monolayers were fixed and stained for tight junction proteins (**H**).

Figure 3. Effect of SP600125 on DSS-induced effects on mouse colon

Adult female mice were fed 2.5% DSS in drinking water with or without intra peritoneal injection of SP600125 (5 mg/kg body weight). Control groups received no treatment or received only SP600125. Body weights (A), stool consistency & rectal bleeding (B) and colon length & weights (C & D) were evaluated. Values are mean $\pm$ SEM ($n = 5-7$).

Asterisk indicates the value that is significantly ($p < 0.05$) different from corresponding control value, and # symbol represents the value that is significantly ($p < 0.05$) different from corresponding DSS without SP600125 value.

Figure 4. DSS-induced neutrophil infiltration in mouse colon is time-dependent

Adult female mice were treated with or without 2.5% DSS in drinking water for 4 or 6 days.

A: Photographs of representative colon on day 4 after DSS treatment. **B:** Formalin-fixed paraffin sections were stained with hematoxylin and eosin and bright field images collected at 10× magnification. **C:** Cryosections of colon were stained for Gr1 (green) and F-actin (Red). **D:** Cryosections of colon were stained for JNK(pT¹⁸³pY¹⁸⁵) (red) and F-actin (green). Staining and imaging are repeated with two more mice for each group with similar results.

Figure 5. DSS disrupts tight junctions by a JNK-dependent mechanism

Adult female mice were fed 2.5% DSS in drinking water with or without intra peritoneal injection of SP600125 (5 mg/kg body weight). Control groups received no treatment or received only SP600126. Cryosections of colon were stained for occludin (green), ZO-1 (red) and nucleus (blue). Staining and imaging are repeated with similar results in two more mice for each group.

Figure 6. DSS disrupts adherens junctions by a JNK-dependent mechanism

Adult female mice were fed 2.5% DSS in drinking water with or without intra peritoneal injection of SP600125 (5 mg/kg body weight). Control groups received no treatment or received only SP600126. Cryosections of colon were stained for E-cadherin (green), β -catenin (red) and nucleus (blue). Staining and imaging are repeated with similar results in two more mice for each group.

Figure 7. Calcium signaling mediates DSS-induced JNK activation and tight junction disruption
A–B: Fura-2 loaded Caco-2 cell monolayers were incubated with 3% DSS in the absence or presence of thapsigargin (TG) or Ca^{2+} -free medium (CFM). Real-time change in $[Ca^{2+}]_i$ was measured and quantitated. Values are mean $\pm$ SEM ($n = 10$). Asterisks indicate the values that are significantly ($p < 0.05$) different from corresponding basal values; symbol # indicates values that are significantly ($p < 0.05$) different from value for DSS group. **C:** Protein extracts from cells treated with 3% DSS in the absence or presence of TG, CFM, or BAPTA for 30 min were immunoblotted for JNK and JNK(pT¹⁸³pY¹⁸⁵) (pJNK). **D–F:** Caco-2 cell monolayers were incubated with 3% DSS in the absence (●) or presence of BAPTA-AM (□), TG (△), or CFM (◇). Control cell monolayers (○) received no treatments. TER (**D**) and inulin permeability (**E**) were measured at varying times. Values are mean $\pm$ SEM ($n = 8$). Asterisks indicate the values that are significantly different ($p < 0.05$) from corresponding control values (○); symbol # indicates values that are significantly ($p < 0.05$) different from value for DSS group (●). Cell monolayers at 2 h after DSS under varying conditions were fixed and stained for tight junction proteins (**F**).

Figure 8. Ask1 and MKK7 are involved in the mechanism of DSS-induced barrier dysfunction and tight junction disruption

A–C: Caco-2 cells were transfected with sets of siRNA for 3 different sites in the gene for human Ask1 (Ask1-siRNA) and MKK7 (MKK7 si-RNA) or non-specific RNA (NS-RNA). Effect of siRNA on Ask1 and MKK7 protein levels was determined by immunoblot analysis (A) and quantitated by densitometric analysis (B & C). Values in panels B and C are mean $\pm$ SE ($n = 3$). Asterisks indicate the values that are significantly different ($p < 0.05$) from corresponding NS-RNA values. **D & E:** Cell monolayers transfected with NS-RNA (○, ●), Ask1-siRNA (□, ■) or MKK7-siRNA (△, ▲) were incubated with (●, ■, ▲ or black bars) or without (○, □, △ or gray bars) DSS and TER (D) was measured at varying times and inulin permeability (E) measured at 3 hours after DSS. Values are mean $\pm$ SE ($n = 4–6$). Asterisks indicate the values that are significantly different ($p < 0.05$) from corresponding control values; symbol # indicates values that are significantly ($p < 0.05$) different from value for NS-RNA DSS group. **F:** At 3 hours after DSS treatment cell monolayers were fixed and stained for occludin (green), ZO-1 (red) and nucleus (blue) by immunofluorescence method and images collected by confocal microscopy.

Figure 9. Calcium and JNK2-mediated c-Src activation is involved in DSS-induced tight junction disruption

A–D: Caco-2 cell monolayers were incubated with 3% DSS for 30 min in the presence or absence of SP600125 (SP), PP2 (Src kinase inhibitor), thapsigargin (TG), or Ca²⁺-free medium (CFM). Protein extracts were immunoblotted for c-Src(pY⁴¹⁸) and total c-Src (**A**) or for JNK and JNK(pT¹⁸³pY¹⁸⁵) (pJNK) (**B**). TER (**C**) and inulin permeability (**D**) were measured in cell monolayers treated with (■, □) or without (●, ○) 3% DSS in the presence (●, ■) or absence (○, □) of PP2. Values are mean $\pm$ SEM ($n = 6$); Asterisks indicate the values that are significantly ($p < 0.05$) different from corresponding control values (○), and the symbol # indicates the values that are significantly ($p < 0.05$) different from corresponding value for DSS group (□). Fixed cell monolayers were stained for occludin and ZO-1 (**E**). **F–I:** Caco-2 cell monolayers were transfected with nonspecific RNA (NS-RNA; ○, □) or c-Src-specific siRNA (●, ■), and incubated with (■, □) or without (●, ○) 3% DSS for varying times. Proteins were immunoblotted for c-Src (**F**). TER (**F**) and inulin permeability (**G**) were measured at varying times. Values are mean $\pm$ SEM ($n = 6$). Asterisks indicate the values that are significantly ($p < 0.05$) different from corresponding control values (■), and the symbol # indicates the values ($p < 0.05$) that are significantly ($p < 0.05$) different from corresponding values for DSS-treated NS-RNA cells (□). Cell monolayers fixed after 2 h of DSS treatment and stained for tight junction proteins (**H**).

Figure 10. DSS induces Tyr-phosphorylation of tight junction and adherens junction proteins by a JNK-dependent mechanism

A–B: Anti-phospho-Tyr immunoprecipitates (IP:p-Tyr) (**A**) and total cell extracts (**B**) from Caco-2 cell monolayers treated with DSS for 2 h with or without SP600125 were immunoblotted for tight junction and adherens junction proteins. **C:** Anti-p-Tyr immunoprecipitates (IP:pY) and total extracts (input) from mucosal extracts of distal colon from mice treated with DSS for 4 days with or without SP600125 administration were immunoblotted for ZO-1.

Figure 11. JNK2 directly phosphorylates c-Src on Thr residues and increases its activity
 Recombinant c-Src (500 ng) was incubated with or without recombinant JNK2 (200 ng) in the presence or absence of ATP (0.5 mM) and in the presence or absence of SP (SP600125) or PP2 in Tris kinase buffer for one hour at 30°C. Samples were then immunoblotted for p-Thr, c-Src(pY418), c-Src or JNK.

Figure 12. Proposed mechanism of DSS-induced tight junction disruption

DSS appears to mobilize calcium from both extracellular medium and endoplasmic reticulum (ER) that triggers Ask1-MKK7-JNK2-cSrc signaling and Tyr-phosphorylation of tight junction and adherens junction proteins leading to disruption of tight junctions.