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Calcium-Mediated Oxidative Stress: a Common Mechanism in Tight Junction Disruption by Different Types of Cellular Stress

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Abstract

The role of reactive oxygen species (ROS) in osmotic stress, dextran sulfate sodium (DSS) and cyclic stretch-induced tight junction disruption was investigated in Caco-2 cell monolayers *in vitro*, and restraint stress-induced barrier dysfunction in mouse colon *in vivo*. Live cell imaging showed that osmotic stress, cyclic stretch and DSS triggered rapid production of ROS in Caco-2 cell monolayers, which was blocked by depletion of intracellular Ca²⁺ by BAPTA. Knockdown of Cav1.3 or TRPV6 channels blocked osmotic stress and DSS-induced ROS production and attenuated tight junction disruption and barrier dysfunction. N-acetyl L-cysteine (NAC) and L-nitroarginine methyl ester (L-NAME) blocked stress-induced tight junction disruption and barrier dysfunction. NAC and L-NAME also blocked stress-induced activation of JNK and c-Src. ROS was co-localized with the mitochondrial marker in stressed cells. Cyclosporin A blocked osmotic stress and DSS-induced ROS production, barrier dysfunction, tight junction disruption and JNK activation. Mitochondria-targeted Mito-TEMPO blocked osmotic stress and DSS-induced barrier dysfunction and tight junction disruption. Chronic restraint stress in mice resulted in the elevation of intracellular Ca²⁺, activation of JNK and c-Src, and disruption of tight junction in the colonic epithelium. Furthermore, corticosterone administration induced JNK and c-Src activation, tight junction disruption and protein thiol oxidation in colonic mucosa. This study demonstrates that oxidative stress is a common signal in the mechanism of tight junction disruption in the intestinal epithelium by different types of cellular stress *in vitro* and bio behavioral stress *in vivo*.

Keywords

Intestine; tight junction; adherens function; barrier function; epithelium; reactive oxygen species; JNK; Src; mitochondria

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Introduction

Chronic stress has been implicated in the pathogenesis of many gastrointestinal diseases, including inflammatory bowel diseases and irritable bowel syndrome [1]. However, the mechanisms involved in the effects of stress in the intestinal epithelium are poorly understood. The loss of colonic mucosal barrier function leading to increased intestinal permeability to macromolecules such as endotoxins is a common factor associated with the pathogenesis of most gastrointestinal diseases [2-4]. Acute restraint stress has been shown to increase gut permeability in rats and mice [5-8], indicating that the mucosal barrier dysfunction leading to increased gut permeability is a likely mechanism involved in stress-induced disease promotion.

Epithelial tight junction (TJ) confers the mucosal barrier function. Disruption of TJ is the primary cause of increased gut permeability to macromolecules. TJ is a multi-protein complex consisting of transmembrane proteins such as occludin, tricellulin, claudins and junctional adhesion molecules, and intracellular adapter proteins such as ZO-1, ZO-2 and ZO-3, which interact with many TJ-specific proteins and the actin cytoskeleton [9-11]. A significant body of evidence indicates that the disruption of intestinal epithelial TJ and elevated gut permeability to macromolecules play crucial role in the pathogenesis of many gastrointestinal diseases [12-15]. Recent studies demonstrated that different types of cellular stress affect the integrity of intestinal epithelial TJ and induce barrier dysfunction [16-19]. Evidence also suggests that acute bio-behavioral stress may affect the TJ in mouse intestinal epithelium *in vivo* [5-8]. Therefore, understanding the mechanisms involved in stress-induced disruption of intestinal epithelial TJ is crucial to develop novel therapeutics to treat stress-related diseases.

Multiple intracellular signaling mechanisms regulate the assembly and disruption of TJ in different epithelia [20]. Recent studies suggested that cell signaling involving intracellular calcium [Ca^{2+}]_i), c-jun N-terminal kinase-2 (JNK2) and c-Src may be involved in TJ disruption by osmotic stress [18, 19], dextran sulfate sodium (DSS) [16] and cyclic stretch in the intestinal epithelium [17]. [Ca^{2+}]_i mediates stress-induced activation of JNK2 and c-Src, but the link between these signaling elements is unknown, as neither JNK2 nor c-Src are unknown to be activated directly by Ca^{2+} . Our previous studies demonstrated that oxidative stress disrupts intestinal epithelial TJ in Caco-2 and MDCK cell monolayers [21-23], raising the question whether oxidative stress is involved in stress-induced TJ disruption.

In the present study, we investigated the potential role of oxidative stress in Ca^{2+} -induced activation of JNK and c-Src, disruption of TJ and barrier dysfunction in Caco-2 cell monolayers. This study also investigates the effects of chronic restraint stress and corticosterone administration on mouse colonic epithelial TJ *in vivo*. The results provide evidence for a common signaling cascade involving [Ca^{2+}]_i-mediated mitochondrial oxidative stress in the disruption of intestinal epithelial TJ by a variety of cellular stress. Furthermore, data implicate that a similar cellular mechanism may be involved in epithelial TJ disruption by chronic restraint stress and corticosterone administration in mouse colon *in vivo*.

Methods and Materials

Chemicals

Cell culture medium (DMEM), fetal bovine serum (FBS) and antibiotics were procured from Cellgro® (Manassas, VA). MitoSOX™, Mito Tracker® Green, CM-H2DCFDA and Hoechst 33342 were purchased from Life Technologies (Grand Island, NY). 1,2-bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid tetrakis (acetoxymethyl) ester (BAPTA-AM) (Sigma Aldrich, St. Louis, MO). N-acetyl L-cysteine (NAC) (Sigma Aldrich, St. Louis, MO). L- N^G nitroarginine methyl ester (L-NAME, Sigma Aldrich, St. Louis, MO). N-(3-(Aminomethyl)benzyl)acetamide (1400W) (Sigma Aldrich, St. Louis, MO). Mito-Vitamin E and Mito-CoQ were synthesized in the laboratory of Dr. Kalyanaraman. Mito-TEMPO (Cayman Chemical company, Ann Arbor, MI). Apocynin (Santa Cruz Biotechnology, Santa Cruz, CA). All other chemicals were purchased from either Sigma Aldrich or Thermo Fisher Scientific (Tustin, CA).

Antibodies

Anti-JNK(pTpY^{183/185}) or pJNK, anti-c-Src(pY⁴¹⁸) or pSrc, anti-ZO-1 and anti-occludin antibodies were purchased from Invitrogen (Carlsbad, CA). Anti-JNK 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, Cy3-conjugated anti-rabbit IgG, AlexaFluor-488 phalloidin and AlexaFluor-546 phalloidin were purchased from Molecular Probes (Eugene, OR). Anti-E-cadherin and anti-β-catenin antibodies were purchased from BD Biosciences (Billerica, MA).

Cell culture

Caco-2_{bbe} cells (American Type Culture Collection, Rockville, MD) were grown under standard cell culture conditions as described before [24]. Experiments were conducted using cells grown in polycarbonate membrane transwell inserts (12 mm diameter) (Costar, Sigma-Aldrich, St. Louis, MO) for 10-15 days.

ShRNA Constructs

Short hairpin RNA specific for human Ca_v1.3 gene was prepared as described before [18]. For human TRPV6 gene silencing, shRNA was designed using the Dharmacon Web site (siDesign® Center, <http://www.dharmacon.com/DesignCenter/>) [Target: GAAACAGCGCTACACATA]. The sequence was further verified by BLAST search by human genome databases, and no matches were found other than TRPV6, confirming the uniqueness of this sequence. To construct shRNA vector, two pairs of oligonucleotides containing the antisense sequence, hair pin loop region (TTGATATCCG) and sense sequence with cohesive BamH1 and Hind III sites were synthesized (Integrated DNA Technologies Inc, Coralville, IA) as follows: top strand, 5'-GATCCCGGAAACAGCGCTACACATATTGATATCCGTATGTGTAGCGCTGTTTCCTTTTTCCTTTTCCAAA-3' and bottom strand, 5'-AGCTTTTGGAAAAAAGGAAACAGCGCTACACATACGGATATCAATATGTGTAGCG

CTGTTTCCGG-3'. The corresponding forward and reverse strands were annealed and inserted at BamHI and HindIII sites into pRNATin-H1.2/Neo (GenScript Corp., Piscataway, NJ) vector, which induces expression of shRNA by H1.2 promoter and cGFP reporter gene by CMV promoter (Promega, WI). The constructs were transformed into DH5 α *E. coli* competent cells for preparation of plasmid DNA. Plasmid DNA were isolated and purified using QIAGEN Plasmid miniprep Kit (Valencia, CA). Insertion of shRNA sequence was confirmed by releasing it by digestion with BamHI and HindIII.

Transfection

Caco-2 cells grown to about 75% confluence were transfected using 1ml of antibiotic and serum-free Opti-MEM[®] containing 1 μ g of plasmid DNA (Vector, Ca_v1.3 or TRPV6-specific shRNA constructs) using Lipofectamine[™] 2000 and Plus reagent in 50 μ l OptiMEM, and incubated for 30 min at room temperature. Transfection mixture was then replaced with normal DMEM containing serum and antibiotics. After 24 hours, cell monolayers were trypsinized, seeded on to Transwell inserts of Costar, Cambridge, MA) and used for experiments on day 3 or 4 after seeding.

Cell treatments

Osmotic stress and DSS treatment—Cell monolayers in transwell inserts were incubated with 3.0% DSS in DMEM or 600 mOsM DMEM (osmolarity adjusted with mannitol) in the apical well.

Cyclic stretch

Caco-2 cell monolayers, grown on collagen coated Flexcell plates, were subjected to cyclic stretch using 12% strain at a frequency of 6 cycles per min using a Flexercell Fx-4000T tension unit (Flexcell International, Hillsborough, NC) for 2-6 hrs. This is a vacuum-driven device that applies biaxial strain to cells regulated by computer-controlled program as explained previously [25]. These conditions of stretch are similar to forces generated due to peristaltic and villus motilities [26, 27]. Control wells were plugged at the bottom by rubber capping without application of any stretch. The inhibitors were present during the cyclic stretch.

Inhibitor and antioxidant treatments

Depletion of [Ca²⁺]_i was achieved by incubation of cells with 10 μ M BAPTA-AM. In some experiments, cell monolayers were incubated with 10 mM NAC, 30 μ M L-NAME, 50 μ M 1400W, 5 μ M diphenyleneiodonium or apocynin (100 μ M) for one hour prior to osmotic stress (600 mOsM), DSS treatment or cyclic stretch. In other experiments, cells were preincubated with Mito-Vitamin E (2.5 μ M), Mito-CoQ (1 μ M), Mito-TEMPO (20 μ M) or Cyclosporin A (CsA; 20 μ M) 30 min prior to osmotic stress or DSS.

Imaging ROS production

Caco-2 monolayers were grown on 60 mm dishes to full confluence. Cell monolayers were washed with DMEM without phenol red were incubated with Hoechst 33342 (1:10000) and 10 μ M CM-H₂DCFDA for 40 min at 37°C. Following a wash, cells were incubated with 5

mM MitoSOX™ for 10 min at 37°C. Cell monolayers were then exposed to osmotic stress, DSS or cyclic stretch for varying times and fluorescence images were captured using a confocal microscope. To determine the mitochondrial association of ROS, cell monolayers were preincubated with 200 nM MitoTracker® green for 30 min followed by incubation with 5 mM MitoSOX™ for 10 min at 37°C and the fluorescence images captured after 30 min.

Epithelial barrier function

Transepithelial electrical resistance (TER) was measured using a Millicell-ERS Electrical Resistance System (Millipore, Bedford, MA). TER was calculated as Ohms.cm² by multiplying it with the surface area of monolayer. To evaluate the paracellular permeability, cell monolayers were incubated with FITC-inulin (6 kD; 0.5 mg/ml) in the apical well and measured the unidirectional flux of FITC-inulin as described previously [19].

Animals

All animal experiments were performed according to the protocols approved by our institutional animal care and use committee. Adult male mice (C57BL/6; 12–14 weeks old) were used in this study. Animals had *ad libitum* access to laboratory chow and water.

Chronic restraint stress in mice

Mice were subjected to restraint stress using a mouse restrainer (Braintree Scientific, Inc., Braintree, MA). Animals restrained daily for 2 hours (9-11 AM) for 1-8 days. Control mice were maintained in the cage without food and water for 2 hours parallel to restrained mice.

Measuring [Ca²⁺]_i in mouse colon *ex vivo*

The colonic strips from mice subjected to restraint stress or sham treatment for varying days were mounted on Sylgard-coated 60 mm dish. The tissue was incubated in a mixture of 20 μM Indo-1 and 0.25% pluronic acid for 1 hour at 37°C in dark. The fluorescence was measured using an upright confocal microscope in two channels at 410 ± 20 nm and 485 ± 20 nm wavelengths. [Ca²⁺]_i was evaluated by ratiometric analysis. In other experiments, mucosal strips from untreated mice were exposed to osmotic stress (600 mOsM) for varying times and [Ca²⁺]_i measured as described above.

Corticosterone treatment

Mice were randomly divided into two groups (n = 6 per group) and injected (*s. c.*) either vehicle (control) or corticosterone (25 mg/kg in sesame oil) daily at 9-10 AM for 7 days. Animals had *ad libitum* access to diet and water. Two hours following the last injection, animals were euthanized and colons were collected for further analysis. The plasma corticosterone level of up to 0.6 μg/ml has been recorded in mice and rats following restraint stress for an hour, which returned to basal level by one hour post stress [28, 29]. In stressed human subjects however, the elevated plasma cortisol is likely to be sustained for much longer period. The dose used in the current study is likely to result in a maximal plasma concentration of 25 μg/ml, which is justified based on that it is a bolus administration rather

than a continuous administration. This dose has been previously used to mimic stress in many studies [30].

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 in 4% nonfat milk in TBST (20 mM Tris, pH 7.2 and 150 mM NaCl) and stained for different proteins as described before [19]. The fluorescence was examined by using a Zeiss LSM 710 laser scanning confocal microscope and 20 $\times$ objective lens. Images from x-y (1 μ m) sections were collected using Zen software. Images from sections were stacked using Image J (National Institute of Health, Bethesda, MD) and processed by Adobe Photoshop (Adobe Systems, San Jose, CA).

Protein thiol assay

Protein thiols in intestinal sections were assessed as described before [25]. Reduced protein thiols were evaluated by staining cryosections of colon with BODIPY FL-*N*-(2-aminoethyl) maleimide (Flm). For oxidized protein thiols, the reduced protein thiol was first alkylated with *N*-ethylmaleimide followed by reduction of oxidized protein thiols with tris (2-carboxyethyl) phosphine prior to Flm staining. Control staining was done after *N*-ethylmaleimide treatment. Fluorescence images were captured and fluorescence density evaluated by densitometric analysis using Image J software.

Immunoblot analysis

Proteins in cell extracts were separated by sodium dodecyl sulfate-polyacrylamide gel (7%) electrophoresis and transferred to polyvinylidene fluoride membranes. The membranes were immunoblotted for different proteins as described before [19]. The blots were developed using the electrochemiluminescence kit (Pierce, Rockford, IL) and quantitated by densitometry using Image J software.

Statistical analysis

All data are expressed as Mean $\pm$ SEM. The differences among multiple groups were first analyzed by ANOVA. When a statistical significance was detected, Tukey's *t* test was used to determine the statistical significance between multiple testing groups and the corresponding control. Statistical significance was established at 95%.

Results

Different types of cellular stress induce rapid production of ROS in Caco-2 cell monolayers by Ca²⁺-dependent mechanism

We first asked the question whether different types of stress induce oxidative stress in the intestinal epithelium. Therefore, ROS generation in response to different types of stress was examined in Caco-2 cell monolayers. MitoSOX™ has been used as a probe for superoxide, whereas DCF is a probe for overall ROS. Osmotic stress rapidly increased MitoSOX™-dependent fluorescence with a maximum level achieved at 30 min, which sustained at least

until 120 min (Fig. 1A). DCF-dependent fluorescence was also increased by osmotic stress, with a maximum level achieved between 60 min and 120 min. MitoSOX™ and DCF-dependent fluorescence were also rapidly elevated by DSS (Fig. 1B) and cyclic stretch (Fig. 1C), with a maximum level achieved at 30 min and sustained until 120 min. Both MitoSOX™ and DCF-dependent fluorescence in DSS and cyclic stretch-treated cells were partially faded during 60-120 min. Depletion of $[Ca^{2+}]_i$ by pretreatment of cell monolayers with BAPTA almost completely blocked osmotic stress, DSS and cyclic stretch-induced elevation of MitoSOX™ and DCF-dependent fluorescence in cells (Fig. 1D). The reason for the partial reduction of fluorescence during 60-120 min of DSS and cyclic stretch treatment is unclear. Osmotic stress and DSS-treated cells also showed fluorescence in the nucleus. The reason for this nuclear localization is unclear at this time. However, MitoSox™ is an ethidium derivative and the ethidium oxidation product is known to bind to DNA emitting red fluorescence. It is therefore likely that oxidized MitoSox may have leaked into the nucleus.

CA_v1.3 and TRPV6 channels are involved in stress-induced ROS production, TJ disruption and barrier dysfunction

To determine the role of calcium channels, Ca_v1.3 and TRPV6, in stress-induced oxidative stress, TJ disruption and barrier dysfunction Caco-2 cells transfected with shRNA constructs. Immunoblot analysis confirmed the specific knockdown of these channels (Fig. 2A). Live cell imaging showed that osmotic stress and DSS-induced ROS production was absent in cell monolayers transfected with Ca_v1.3 or TRPV6, whereas vector-transfected cell monolayers showed ROS production (Fig. 2B). Osmotic stress-induced decrease in TER (Fig. 2C) and increase in inulin permeability (Fig. 2D) were significantly attenuated in cell monolayers transfected with Ca_v1.3 or TRPV6-specific shRNA constructs. Similarly, DSS-induced decrease in TER (Fig. 2E) and increase in inulin permeability (Fig. 2F) were attenuated in cell monolayers transfected with Ca_v1.3 or TRPV6-specific shRNA constructs. Transfection of Ca_v1.3 or TRPV6-specific shRNA constructs also attenuated osmotic stress and DSS-induced redistribution of occludin and ZO-1 from the intercellular junctions (Fig. 2G)

Antioxidants attenuate osmotic stress and DSS-induced barrier dysfunction in Caco-2 cell monolayers

To determine the role of ROS in stress-induced barrier dysfunction in Caco-2 cell monolayers we evaluated the effects of different antioxidants on stress-induced barrier dysfunction. Pretreatment of cell monolayers with NAC significantly attenuated osmotic stress-induced decrease in TER (Fig. 3A) and inulin permeability (Fig. 3B). Pretreatment with L-NAME, but not 1400W, also significantly blocked osmotic stress-induced decrease in TER (Fig. 3A) and inulin permeability (Fig. 3B). Similarly, NAC and L-NAME, but not 1400W blocked DSS-induced decrease in TER (Fig. 3C) and increase in inulin permeability (Fig. 3D). Pretreatment of cells with apocynin or diphenyleneiodonium, the inhibitors of NADPH oxidase, failed to block osmotic stress or DSS-induced changes in TER or inulin permeability (data not shown).

Antioxidants block stress-induced disruption of TJ and adherens junction (AJ)

To determine the role of ROS in stress-induced disruption of TJ we examined TJ integrity by immunofluorescence staining of cell monolayers for occludin and ZO-1. Osmotic stress (Fig. 3A), DSS (Fig. 3B) and cyclic stretch (Fig. 3C) induced redistribution of occludin and ZO-1 from the intercellular junctions into the intracellular compartments. Pretreatment of cell monolayers with NAC blocked the effects of osmotic stress, DSS and cyclic stretch on TJ protein redistribution. L-NAME, but not 1400W, also attenuated osmotic stress, DSS and cyclic stretch-induced redistribution of occludin and ZO-1, but this effect was only partial in DSS and cyclic stretch-treated cell monolayers.

AJ was examined by staining the cell monolayers for E-cadherin and β -catenin. NAC treatment blocked osmotic stress (Fig. 5A), DSS (Fig. 5B) and cyclic stretch (Fig. 5C)-induced redistribution of E-cadherin and β -catenin from the intercellular junctions. Once again, L-NAME, but not 1400W partially attenuated osmotic stress, DSS and cyclic stretch-induced redistribution of E-cadherin and β -catenin.

Antioxidants attenuate stress-induced activation of JNK and c-Src

The role of $[\text{Ca}^{2+}]_i$ -mediated oxidative stress in stress-induced TJ and AJ disruption raised the question whether oxidative stress mediates stress-induced activation of JNK and c-Src. Therefore, we evaluated the effect of antioxidants on osmotic stress, DSS and cyclic stretch-induced activation of JNK and c-Src. Immunoblot analysis showed that osmotic stress (Fig. 6A), DSS (Fig. 6B) and cyclic stretch (Fig. 6C) increased the levels of pJNK and pSrc in Caco-2 cell monolayers. Pretreatment of cells with NAC or L-NAME attenuated osmotic stress, DSS and cyclic stretch-induced increase in pJNK and pSrc. 1400W failed to affect stress-induced increase in pJNK or pSrc. Elevation of pJNK by oxidative stress and its attenuation by NAC and L-NAME was further confirmed by immunofluorescence microscopy for pJNK (Fig. 6D). Fluorescence images also show that osmotic stress-induced disruption of actin cytoskeleton was blocked by NAC and partially attenuated by L-NAME, but not by 1400W.

Role of mitochondrial oxidative stress in osmotic stress and DSS-induced barrier dysfunction and TJ disruption

In order to determine the subcellular source of oxidative stress, we co-stained cells for superoxide with MitoSOXTM and mitochondria with MitoTracker[®]. MitoSOXTM fluorescence was found to be co-localized with MitoTracker[®] in osmotic stress, DSS and cyclic stretch-treated cells (Fig. 7A). Osmotic stress and DSS-induced ROS production was almost completely blocked by pretreatment of cells with CsA (Fig. 7B). The effect of CsA on osmotic stress and DSS-induced changes in TER and inulin permeability was evaluated to determine the role of mitochondrial membrane permeability transition (MMPT) in barrier disruption. Pretreatment of cell monolayers with CsA completely blocked osmotic stress and DSS-induced decrease in TER (Fig. 7C) and increase in inulin permeability (Fig. 7D). CsA by itself did not produce significant effect on TER or inulin flux. CsA treatment also attenuated osmotic stress and DSS-induced redistribution of occludin and ZO-1 from the intercellular junctions (Fig. 7E). In the absence of CsA, osmotic stress and DSS elevated

pJNK and induced reorganization of actin cytoskeleton (Fig. 7F), which was blocked by pretreatment of cells with CsA (Fig. 7G).

Mitochondria-targeted superoxide scavenger attenuates osmotic stress and DSS-induced barrier dysfunction and TJ disruption

The potential role of mitochondrial oxidative stress in disruption of TJ and barrier dysfunction by different types of stress raised the question whether mitochondria-targeted antioxidants are effective in preventing stress-induced barrier dysfunction and TJ disruption. We evaluated the effects of Mito-vitamin E, Mito-CoQ and Mito-TEMPO on osmotic stress and DSS-mediated TJ disruption and barrier dysfunction. Pretreatment of cell monolayers with Mito-CoQ failed to affect osmotic stress and DSS-induced decrease in TER (Fig. 8A & 8C), increase in inulin permeability (Fig. 8B & 8D) or redistribution of occludin and ZO-1 from the intercellular junctions (Fig. 8E). Mito-Vitamin E pretreatment caused no significant effect on osmotic stress-induced decrease in TER (Fig. 8A), inulin permeability (Fig. 8B) and redistribution of occludin and ZO-1 (Fig. 8E), but it significantly blocked DSS-induced decrease in TER (Fig. 8C) and increase in inulin permeability (Fig. 8D), with a partial prevention of occludin and ZO-1 redistribution (Fig. 8E).

Interestingly, pretreatment of cell monolayers with Mito-TEMPO ablated osmotic stress and DSS-induced decrease in TER (Fig. 8F) and increase in inulin permeability (Fig. 8G). Mito-TEMPO also completely blocked osmotic stress and DSS-induced redistribution of occludin and ZO-1 from the intercellular junctions (Fig. 8H).

Restraint stress and corticosterone administration activate Ca^{2+} /JNK/c-Src signaling and TJ disruption in mouse colon *in vivo*

Data described above indicate that $[\text{Ca}^{2+}]_i$ -mediated mitochondrial oxidative stress is involved in stress-induced activation of JNK and c-Src in Caco-2 cell monolayers *in vitro*. These data raised the question whether a similar signaling cascade is activated by biobehavioral stress *in vivo*. We examined the effect of chronic restraint stress on $[\text{Ca}^{2+}]_i$ /JNK/c-Src signaling in mouse colon. Animals were subjected to 2-hour restraint stress daily for 8 days. All analyses were performed immediately after 2-hour stress on a particular day of chronic restraint stress. $[\text{Ca}^{2+}]_i$ was measured in colonic strips prepared from mice subjected to stress on different days using Indo-1 as a probe, and the fluorescence images were captured by a confocal microscope (Fig. 9A). Quantitation by ratiometric analysis indicated that chronic restraint stress increased the level of $[\text{Ca}^{2+}]_i$ in the colonic epithelial cells beginning 4 days of stress (Fig. 9B). A similar increase in $[\text{Ca}^{2+}]_i$ was recorded when colonic strips from control mice were exposed to osmotic stress *ex vivo* (Fig. 9C & 9D). Confocal microscopy of cryosections of colon from control mice and mice stressed for 2 hours on day 5 showed an elevation of pJNK and pSrc in the colon (Fig. 9E). Confocal microscopy for occludin and ZO-1 showed that chronic restraint stress induced redistribution of these TJ proteins from the intercellular junctions of colonic epithelium in a time-dependent manner, with considerable disruption detected on day 4 and a progressive disruption until day 8 (Fig. 9F).

A major mechanism of biobehavioral stress-induced pathophysiology is elevation of plasma glucocorticoid via Hypothalamo-Pituitary-Adrenal (HPA) axis [31]. Corticosterone, the rodent glucocorticoid, has been shown to be elevated during chronic restraint stress [32]. To mimic chronic restraint stress we administered corticosterone to mice daily for 7 days and examined the effect of corticosterone on the actin cytoskeleton, JNK/c-Src activation, TJ protein distribution and oxidative stress. Corticosterone increased the levels of pJNK and pSrc in mouse colon (Fig. 9G, 9H & 9I). The F-actin stain (Fig. 9G) and junctional distribution of ZO-1 (Fig. 9H) were dramatically low in corticosterone treated mouse colon compared to that in the control mouse colon. To determine the effect of corticosterone on oxidative stress we examined its effect on the levels of reduced and oxidized protein thiols by fluorescence microscopy. Corticosterone treatment depleted the reduced protein thiols, with a concomitant increase in the levels of oxidized protein thiols (Fig. 9J & 9K).

Discussion

Identifying the signaling mechanisms involved in the disruption of epithelial TJ by various types of stress is essential not only to understand the pathogenesis of various gastrointestinal and related diseases, but also to develop novel therapeutics to treat diseases by targeting multiple injurious factors with a common therapeutic regimen. Osmotic stress, chemical injury and excessive stretch of the intestinal wall are some of the injurious factors involved in the pathogenesis of gastrointestinal diseases. Our study provides evidence supporting that calcium-mediated mitochondrial oxidative stress leading to activation of JNK-c-Src signaling is a common mechanism involved in TJ disruption in the intestinal epithelium by osmotic stress, DSS and cyclic stretch in Caco-2 cells and bio behavioral stress in mice. This study demonstrates that oxidative stress links the rise in $[Ca^{2+}]_i$ with the activation of JNK-c-Src signaling pathway.

The increase in MitoSOXTM and DCF-dependent fluorescence indicates that osmotic stress, DSS and cyclic stretch induce a rapid increase in ROS production in Caco-2 cells. MitoSOXTM is considered as a specific probe for mitochondrial superoxide [33]. However, as described in the results section MitoSOXTM may have leaked into the nucleus, thus the source of this ROS is unclear. DCF is known to detect most ROS with less specificity. All types of cellular stress used in this study generated a maximum level of MitoSOXTM-dependent fluorescence by 30 min and maximum level of DCF-dependent fluorescence achieved during 60-120 min. The reason for gradual reduction of MitoSOXTM-dependent and DCF-dependent fluorescence in DSS and cyclic stretch-treated cells is unclear. Some of the possibilities include quenching of fluorescence or leak of oxidized probes into extracellular medium. Overall, the data demonstrate that different types of cellular stress induce rapid production of ROS in Caco-2 cells. Interestingly, the present study shows that ROS production by osmotic stress, DSS and cyclic stretch is abolished by the depletion of $[Ca^{2+}]_i$. Our previous studies showed that osmotic stress and DSS induce a rapid elevation of $[Ca^{2+}]_i$ involving both ER Ca^{2+} release and influx of extracellular Ca^{2+} [16, 18]. The present study demonstrates that $[Ca^{2+}]_i$ mediates stress-induced ROS production.

Our previous study showed that the Ca^{2+} channel $Ca_v1.3$ plays an important role in osmotic stress-induced TJ disruption and barrier dysfunction [18]. Intestinal epithelium is known to

express both $\text{Ca}_v1.3$ and TRPV6 channels on the apical membrane, and therefore we investigated the role of these two channels in stress-induced ROS production and TJ disruption. Specific knockdown of $\text{Ca}_v1.3$ or TRPV6 channels blocked osmotic stress and DSS-induced ROS production and attenuated TJ disruption and barrier dysfunction. These data confirm the role of $\text{Ca}_v1.3$ channel in TJ disruption, but also demonstrate that it is required for stress-induced ROS production. Furthermore, the current study demonstrates that TRPV6 channel is also required for osmotic stress and DSS-induced ROS production and TJ disruption. It is unclear why both channels are required for the stress effects. It is likely that these two channels play synergistic role in elevating $[\text{Ca}^{2+}]_i$ and therefore ROS production and TJ disruption. TRPV6 channel was originally cloned and characterized as channel-like transporter mediating intestinal Ca^{2+} absorption [34]. Since then it has been studied as a vitamin D-regulated transporter in intestinal absorption of Ca^{2+} . The current study provides evidence for cell signaling role of intestinal TRPV6 channel.

Ca^{2+} -mediated ROS production raised the question whether oxidative stress played a role in stress-induced disruption of TJ and barrier dysfunction. Attenuation of osmotic stress and DSS-induced decrease in TER and increase in inulin permeability by NAC and L-NAME indicate that oxidative stress does play a role in stress-induced barrier dysfunction in the intestinal epithelium. Barrier dysfunction by osmotic stress and DSS was unaffected by 1400W or apocynin. L-NAME is a general nitric oxide synthase (NOS) inhibitor, whereas 1400W is more specific to inducible NOS (iNOS). The lack of an effect by 1400W suggest that iNOS is not involved in osmotic stress or DSS-induced epithelial barrier dysfunction. Apocynin is a selective inhibitor of NADPH oxidase [35] and a lack of its effect on osmotic stress and DSS-induced barrier dysfunction suggests that NADPH oxidase-mediated ROS production is not involved in stress-induced effects. Attenuation of occludin, ZO-1, E-cadherin and β -catenin redistribution indicates that NAC and L-NAME block osmotic stress, DSS and cyclic stretch-induced disruption of TJ and AJ. Once again, 1400W and apocynin were ineffective in stress-induced disruption of TJ and AJ. These data demonstrate that Ca^{2+} -induced ROS production is a key mediator in TJ disruption and barrier dysfunction by various types of cellular stress. Two of the models of stress-induced TJ disruption apply to other epithelia. Studies by other laboratories have shown that cyclic stretch disrupts TJ in the alveolar epithelium [36] and osmotic stress causes TJ disruption in airway epithelium and blood brain barrier [37, 38]. There is no reported study on the effect of DSS on TJ in other epithelia as it is relevant only to the intestinal epithelium due to its role in inducing colitis.

Previous studies showed that $[\text{Ca}^{2+}]_i$ -induced activation of JNK and c-Src played an important role in osmotic stress and DSS-induced TJ disruption in Caco-2 cells and mouse colon [16, 18, 19]. This raised the question whether stress-induced oxidative stress played a role in activation of JNK and c-Src. Elevation of pJNK and pSrc confirm that JNK and c-Src are activated by osmotic stress, DSS and cyclic stretch in Caco-2 cells. Attenuation of JNK and c-Src activation by NAC and L-NAME indicate that oxidative stress plays an important role in JNK and c-Src activation by osmotic stress, DSS and cyclic stretch. Apocynin and 1400W failed to affect JNK and c-Src activation by different cellular stresses. These data indicate that $[\text{Ca}^{2+}]_i$ -mediated oxidative stress activates JNK and c-Src, which in turn may disrupt TJ in the intestinal epithelium. Our previous studies showed that JNK activates c-Src,

and c-Src regulates TJ integrity by tyrosine phosphorylation of occludin leading to dissociation of ZO-1.

Mitochondrial oxidative stress plays an important role in cell injury. While mitochondrial oxidative stress is triggered by multiple factors, rise in $[Ca^{2+}]_i$ appears to be involved in MMPT and ROS production [39, 40]. A significant co-localization of MitoSOXTM-dependent fluorescence with MitoTracker[®] suggests that mitochondria may play a role in osmotic stress, DSS and cyclic stretch-mediated ROS production. CsA, an MMPT blocker, abolished osmotic stress and DSS-mediated ROS production, indicating that MMPT plays a major role in stress-induced production of ROS. A significant attenuation of changes in TER, inulin permeability and redistribution of occludin and ZO-1 by CsA demonstrate that MMPT is involved in osmotic stress and DSS-induced barrier dysfunction and TJ disruption. CsA also blocked osmotic stress and DSS-induced activation of JNK and c-Src. Data demonstrate that MMPT and mitochondrial oxidative stress play a crucial role in stress-induced TJ disruption.

Role of mitochondria in Ca^{2+} -induced oxidative stress and TJ disruption raised the question whether or not mitochondria-targeted antioxidants are effective in preventing stress-induced barrier dysfunction and TJ disruption. Mito-vitamin E, Mito-CoQ and Mito-TEMPO are mitochondria targeted antioxidants. Mito-vitamin E and Mito-CoQ were not effective in preventing either osmotic stress or DSS-induced TJ disruption or barrier dysfunction. But, Mito-vitamin E showed a partial effect on DSS-induced TJ disruption and barrier dysfunction. On the other hand, Mito-TEMPO completely blocked TJ disruption and barrier dysfunction by both osmotic stress and DSS, indicating that production of superoxide in the mitochondria plays an important role in osmotic stress and DSS-induced TJ disruption. Mito-TEMPO is a mitochondria targeting derivative of tempol, a superoxide scavenger [41]. Vitamin E exerts antioxidant activity by scavenging hydroxyl radicals. Our previous study showed that oxidative stress-induced TJ disruption in Caco-2 cell monolayers involves hydrogen peroxide, but not hydroxyl radicals [42], which explain why Mito-vitamin E was not effective in blocking stress-induced TJ disruption. These results indicate that mitochondria-targeted superoxide scavengers are highly effective in preventing stress-induced oxidative stress and epithelial barrier dysfunction.

The role of $[Ca^{2+}]_i$, mitochondrial oxidative stress, JNK and c-Src in TJ disruption by different types of cellular stress in Caco-2 cell monolayers indicates that a common signaling mechanism is involved in stress-induced disruption of intestinal epithelial TJ and barrier dysfunction. This interpretation raised the question whether a similar signaling mechanism is activated during bio-behavioral stress-induced TJ disruption in colonic epithelium *in vivo*. The present study shows that chronic restraint stress for more than 3 days causes a significant increase in $[Ca^{2+}]_i$ in mouse colonic epithelial cells. Elevated $[Ca^{2+}]_i$, which was associated with a redistribution of occludin and ZO-1 from the intercellular junctions of colonic epithelium, indicating that TJ disruption is in parallel to the elevation of $[Ca^{2+}]_i$. TJ disruption and elevation of $[Ca^{2+}]_i$ by chronic restraint stress was associated with a robust increase in the levels of activated JNK and c-Src in the colonic epithelial cells.

Biobehavioral stress is known to increase plasma corticosterone via HPA axis [31]. To determine the mechanism of restraint stress-induced TJ disruption and activation of JNK and c-Src, we evaluated the effect of chronic corticosterone administration on colonic epithelium in mice. Corticosterone induced a robust increase in pJNK and pSrc and caused redistribution of ZO-1 from the intercellular junctions of colonic epithelium. Activation of JNK and c-Src and TJ disruption by restraint stress and corticosterone administration suggest that glucocorticoid-mediated activation of $[Ca^{2+}]_i$ /JNK/c-Src signaling pathway may be involved in restraint stress-induced disruption of colonic epithelial TJ and increased permeability to macromolecules. Interestingly, corticosterone administration caused a dramatic increase in protein thiol oxidation in colonic mucosa, which is consistent with the stress-induced oxidative stress in Caco-2 cells. Therefore, oxidative stress is an important component of signaling mechanism in stress-induced disruption of colonic epithelial TJ.

In summary, this study demonstrates that $Ca_v1.3$ and TRPV6 channels and $[Ca^{2+}]_i$ -mediated MMPT and mitochondrial oxidative stress play a crucial role in TJ disruption and barrier dysfunction in Caco-2 cell monolayers by different cellular stress. Furthermore, study provides evidence suggesting that a similar signaling mechanism may be involved in restraint stress-induced TJ disruption in mouse colon *in vivo*. Pathogenesis of gastrointestinal diseases involves multiple types of stress, including osmotic stress, chemical injury and excessive bloating of intestine. Identifying a common mechanism in the effects of different types of cellular stress offers a common target to develop therapeutics for treatment of gastrointestinal diseases.

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Abbreviations

AJ	adherens junction
BAPTA-AM	1,2-bis(<i>o</i> -aminophenoxy)ethane- <i>N,N,N',N'</i> -tetra acetic acid tetra(acetoxymethyl) ester
[Ca²⁺]_i	intracellular calcium
CRS	chronic restraint stress
CsA	cyclosporin-A

DSS	dextran sodium sulfate
ER	endoplasmic reticulum
FITC	fluorescein isothiocyanate
CM-H₂DCF-DA	chloromethyl derivative of 2',7' dichlorodihydrofluorescein diacetate
HRP	horse radish peroxidase
JNK	c-Jun N-terminal kinase
L-NAME	L-N ^G nitroarginine methyl ester
NOS	nitric oxide synthase
NAC	N-acetyl L-cysteine
PT	protein thiol
ROS	reactive oxygen species
TBST	tris buffered saline with tween 20
TER	transepithelial electrical resistance
TJ	tight junction
ZO-1	zonula occludens-1
1400W	N-(3-(Aminomethyl)benzyl acetamidine

Summary Statement

We identified a common mechanism, involving calcium, oxidants and protein kinases like JNK and c-Src, in breakdown of gut epithelium by osmotic, chemical and mechanical stress. This opens a common target for blocking epithelial breakdown by different types of stress.

Fig. 1. Different types of stress induce rapid production of ROS in Caco-2 cell monolayers by a $[Ca^{2+}]_i$ -dependent mechanism

A-C: Cell monolayers were preloaded with MitoSOXTM, DCF-DA and Hoechst 33342 forty min prior to exposure to osmotic stress (**A**), DSS (**B**) or cyclic stretch (**C**) for varying times and fluorescence images collected for MitoSOXTM (red), DCF (green) and nucleus (blue) by confocal microscopy. **D:** Cell monolayers were preloaded with MitoSOXTM, DCF-DA, and Hoechst 33342 with or without BAPTA forty min prior to exposure to osmotic stress, DSS

or cyclic stretch. Fluorescence images for MitoSOX™ (red), DCF (green) and nucleus (blue) were captured simultaneously. All experiments were repeated at least two times.

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Fig. 2.

A: Caco2 cells were transfected with Ca_v1.3 or TRPV6-specific shRNA constructs or vector and protein extracts from transfected cells were immunoblotted using specific antibodies. **B:** MitoSOXTM (red) and DCF (green)-sensitive ROS production was detected by live cell fluorescence imaging in Caco-2 cell monolayers transfected with the vector or Ca_v1.3 or TRPV6-specific shRNA constructs at 30 min exposure to osmotic stress (OS) or DSS. Experiments were repeated in at least two monolayers. **C-F:** Vector (■), Ca_v1.3 shRNA (▲) or TRPV6 (◆) shRNA-transfected cell monolayers were incubated with (closed symbols) or

without (○) OS (C & D) or DSS (E & F). TER (C & E) and inulin permeability (D & F) were measured at varying times. Values are mean $\pm$ SEM (n = 4). Asterisks indicate OS or DSS values that are significantly different ($p < 0.05$) from corresponding values for Ca_v1.3 or TRPV6 cell monolayers. **G:** Vector, Ca_v1.3 shRNA or TRPV6 shRNA-transfected cell monolayers were incubated with or without OS or DSS. At 3-hour treatment, cell monolayers were fixed and stained for occludin (green), ZO-1 (red) and nucleus (blue). Analyses were repeated in at least two more monolayers.

Fig. 3. Antioxidants attenuate stress-induced barrier dysfunction in Caco-2 cell monolayers
 Cell monolayers in transwells were pretreated with (gray symbols) or without (closed circles), NAC (triangles), L-NAME (diamonds) or 1400W (squares) prior to exposure to osmotic stress (A & B) or DSS (C & D). Control cell monolayers received no treatments (open circles). TER (A & C) and inulin flux (B & D) 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. Hash tags indicate the values that are significantly ($p < 0.05$) different from corresponding osmotic stress or DSS values.

Fig. 4. Antioxidants block stress-induced TJ disruption in Caco-2 cell monolayers

Cell monolayers in transwells were pretreated with or without, NAC, L-NAME or 1400W prior to exposure to osmotic stress (OS; A), DSS (B) or cyclic stretch (CS; C). After 3 hours, cell monolayers were fixed and stained for occludin (green) and ZO-1 (red). Gray scale images provided only for ZO-1 and images for occludin are similar to that for ZO-1. Analyses were repeated in at least two more monolayers.

Fig. 5. Antioxidants block stress-induced AJ disruption in Caco-2 cell monolayers

Cell monolayers in transwells were pretreated with or without, NAC, L-NAME or 1400W prior to exposure to osmotic stress (OS; **A**), DSS (**B**) or cyclic stretch (CS; **C**). After 3 hours, cell monolayers were fixed and stained for E-cadherin (green) and β -catenin (red). Gray scale images are provided only for E-cadherin. Gray scale images provided only for E-cadherin and images for β -catenin are similar to that for E-cadherin. Analyses were repeated in at least two more monolayers.

Fig. 6. Antioxidants block stress-induced activation of JNK and c-Src in Caco-2 cell monolayers
A-C: Cell monolayers in transwells were pretreated with or without, NAC, L-NAME or 1400W prior to exposure to osmotic stress (OS; **A**), DSS (**B**) or cyclic stretch (CS; **C**). At 30 min, extracts from cell monolayers were immunoblotted for pJNK, pSrc, JNK, c-Src and actin. These experiments were repeated twice with similar results. **D:** Cell monolayers in transwells were pretreated with or without, NAC, L-NAME or 1400W prior to exposure to osmotic stress (OS). After 30 min, cell monolayers were fixed and stained for F-actin (red) and pJNK (green). All experiments were repeated in at least two times.

Fig. 7. Role of mitochondria in stress-induced barrier dysfunction, TJ disruption, ROS production and JNK activation

A: Caco-2 cell monolayers were loaded with MitoSOX™ and MitoTracker®. After 30 min, cells exposed to osmotic stress (OS), DSS or cyclic stretch (CS) for 30 min. Images for MitoSOX™ (red), MitoTracker® (green) and nucleus (blue) were captured by confocal microscopy. **B:** Caco-2 cell monolayers preloaded with MitoSOX™ and DCF-DA and preincubated with or without CsA were exposed to OS or DSS. At 30 min after onset of OS or DSS treatment, fluorescence was imaged for MitoSOX™ (red), DCF (green) and nucleus

(blue). **C-E:** Cell monolayers on transwells were preincubated with (closed symbols) or without (open symbols) CsA 30 min prior to onset of osmotic stress (OS; triangles, green lines), DSS (squares, blue lines) or control treatment (circles, black lines). TER (C) and inulin flux (D) 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. Hash tags indicate the values that are significantly ($p < 0.05$) different from corresponding OS or DSS values. At 3 hours, fixed cell monolayers were stained for occludin (green), ZO-1 (red) and nucleus (blue) (E). **F & G:** Cell monolayers preincubated with (G) or without CsA (F) for 30 min and exposed to OS or DSS for 30 min prior to fixation and staining for F-actin (green) and pJNK (red). All imaging analyses were repeated in at least two more monolayers.

Fig. 8. Effects of mitochondria-targeted antioxidants on osmotic stress and DSS-induced barrier dysfunction and TJ disruption

A-E: Caco-2 cell monolayers were preincubated with (open symbols) or without (closed symbols) Mito-Vitamin E (▲, △, green lines) or Mito-CoQ (■, □, blue lines) for 30 min prior to treatment with osmotic stress (A & B) or DSS treatment (C & D). Control cell monolayers received no treatments (●, ○). TER (A & C) and inulin permeability (B & D) were measured at varying times. Values are mean ± SEM (n = 4-6). Asterisks indicate OS or DSS values that are significantly different (p<0.05) from corresponding values for control

cell monolayers. Hash tags indicate the values for OS and DSS with antioxidant that are significantly different ($p < 0.05$) from corresponding values for OS and DSS without antioxidant. At 3-hour treatment, cell monolayers were fixed and stained for occludin (green), ZO-1 (red) and nucleus (blue) (E). **F-H:** Caco-2 cell monolayers were preincubated with (closed symbols) or without (open symbols) Mito-TEMPO for 30 min prior to treatment with osmotic stress (triangles, green lines) or DSS treatment (squares, blue lines). Control cell monolayers received no treatments (circles, black lines). TER (F) and inulin permeability (G) were measured at varying times. Values are mean $\pm$ SEM ($n = 4-6$). Asterisks indicate that OS or DSS values that are significantly different ($p < 0.05$) from corresponding values for control cell monolayers. Hash tags indicate the OS and DSS with antioxidant values that are significantly different ($p < 0.05$) from corresponding values for OS and DSS without antioxidant. At 3-hour treatment, cell monolayers were fixed and stained for occludin (green), ZO-1 (red) and nucleus (blue) (H). All imaging analyses were repeated in at least two more monolayers.

Fig. 9. Chronic restraint stress and corticosterone administration activate Ca^{2+} /JNK/c-Src signaling, induce oxidative stress and disrupt TJ integrity in mouse colon

A-D: Adult mice were subjected to chronic restraint stress (CRS) for varying times (**A**). Colonic strips (immediately following last cycle of restraint stress) were mounted to Sylgard-coated plates, loaded with Indo-1 for 30 min and fluorescence measured at two channels as described in Methods (**A**) and the fluorescence ratio recorded (**B**). In another set of experiments, mucosal strips from untreated mice loaded with Indo-1 as above were exposed to osmotic stress for varying times (**C** & **D**). Fluorescence images were captured

(C) and quantitated by ratiometric analysis as above (D). Values are mean $\pm$ SEM (n = 4). Asterisks indicate the values that are significantly (p<0.05) different from corresponding 0-Day or 0-min values. **E & F:** Cryosections of distal and/or proximal colons from mice subjected to CRS for 5 days (E) or varying times (F) were stained for F-actin and pJNK or pSrc (E), or occludin and ZO-1 (F). **G-J:** Adult mice were injected with corticosterone daily for 7 days. Cryosections of distal colon were stained for F-actin (green), pJNK (red) and nucleus (blue) (G) or ZO-1 (green), pSrc (red) and nucleus (blue) (H). Density of pJNK and pSrc fluorescence was measured in vehicle (Veh) and corticosterone (Cort.) treated mouse colon (I). Values are mean $\pm$ SEM (n = 4). Asterisk indicates the value that is significantly (p<0.05) different from corresponding Veh value. Cryosections of colons from vehicle or corticosterone (Cort.) were also stained for reduced (Red.-PT) and oxidized (Ox.-PT) protein thiols. Fluorescence images collected using a confocal microscope (J). Background (Bkgd) fluorescence was captured as described in Methods. Density of Red.-PT and Ox.-PT fluorescence was measured in vehicle (Veh) and corticosterone (Cort.) treated mouse colon (K). Values are mean $\pm$ SEM (n = 4). Asterisk indicates the value that is significantly (p<0.05) different from corresponding Veh value. All imaging analyses were repeated in at least two more monolayers.