Roles of ZO-1, occludin, and actin in oxidant-induced barrier disruption

Mark W. Musch,* Margaret Mary Walsh-Reitz,* and Eugene B. Chang

The Martin Boyer Laboratories, Inflammatory Bowel Disease Research Center, Department of Medicine, The University of Chicago, Chicago, Illinois

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Musch, Mark W., Margaret Mary Walsh-Reitz, and Eugene B. Chang. Roles of ZO-1, occludin, and actin in oxidant-induced barrier disruption. Am J Physiol Gastrointest Liver Physiol 290: G222–G231, 2006. First published October 20, 2005; doi:10.1152/ajpgi.00301.2005.—Oxidants such as monochloramine (NH₂Cl) decrease epithelial barrier function by disrupting perijunctional actin and possibly affecting the distribution of tight junctional proteins. These effects can, in theory, disturb cell polarization and affect critical membrane proteins by compromising molecular fence function of the tight junctions. To examine these possibilities, we investigated the actions of NH₂Cl on the distribution, function, and integrity of barrier-associated membrane, cytoskeletal, and adaptor proteins in human colonic Caco-2 epithelial monolayers. NH₂Cl causes a time-dependent decrease in both detergent-insoluble and -soluble zonula occludens (ZO)-1 abundance, more rapidly in the former. Decreases in occludin levels in the detergent-insoluble fraction were observed soon after the fall of ZO-1 levels. The actin depolymerizer cytochalasin D resulted in a decreased transepithelial resistance (TER) more quickly than NH₂Cl but caused a more modest and slower reduction in ZO-1 levels and in occludin redistribution. No changes in the cellular distribution of claudin-1, claudin-5, or ZO-2 were observed after NH₂Cl. However, in subsequent studies, the immunofluorescent cellular staining pattern of all these proteins was altered by NH₂Cl. The actin-stabilizing agent phalloidin did not prevent NH₂Cl-induced decreases in TER or increases of apical to basolateral flux of the paracellular permeability marker mannitol. However, it partially blocked changes in ZO-1 and occludin distribution. Tight junctional fence function was also compromised by NH₂Cl, observed as a redistribution of the α-subunit of basolateral Na⁺K⁺-ATPase to the apical membrane, an effect not found with the apical membrane protein Na⁺/H⁺ exchanger isoform 3. In conclusion, oxidants not only disrupt perijunctional actin but also cause redistribution of tight junctional proteins, resulting in compromised intestinal epithelial barrier and fence function. These effects are likely to contribute to the development of malabsorption and dysfunction associated with mucosal inflammation of the digestive tract.

oxidents; actin cytoskeleton; tight junctions; transepithelial electrical resistance

Barrier function is an essential task of all epithelia dependent on both the integrity and contribution of many cytoskeletal and membrane proteins that comprise and regulate tight junctional complexes that exist between cells (10, 14, 32, 39). Molecular fence function is the property of tight junctions important for maintaining segregation of membrane proteins in polarized epithelia. Barrier function is highly regulated, allowing the epithelium to control transmucosal permeability to solutes, water, and electrolytes (2, 23, 30, 37). The tight junctional complex comprises a large number of membrane-associated and membrane proteins, the latter including occludin, junction adhesion molecule (JAM), and claudins (3, 6, 7, 9), which are responsible for forming the physical connections between cells that confer the basic barrier properties. Occludin has been studied extensively and has a large cytoplasmic extension that may coordinate with junctional proteins in the cytosol (3, 25, 37, 39). Occludin associates with the first tight junction-associated protein identified, zonula occludens-1 (ZO-1) (41). ZO-1 interacts not only with occludin but also with a number of other tight junction-associated proteins including ZO-2, ZO-3, β-catenin, paxillin, talin, and the perijunctional actin ring (11, 39, 44).

The perijunctional actin ring is also important for maintaining barrier function, as the disruption of actin cytoskeleton by cytochalasin causes increased permeability to small solutes (20, 22, 23). Because ZO-1 serves as an important linker molecule between perijunctional actin and occludin, annular contraction in perijunctional actin can dynamically affect intercellular permeability.

In addition to their importance in regulating the movement of solutes in the paracellular pathway, tight junctions also play an important role in the maintenance of cell polarity. This fence function limits movement of proteins and lipids from the apical to basolateral (and vice versa) poles of cells (15, 16, 24). The ability of the junctional complexes to regulate the fence function may be compromised by a number of stimuli including Ca2⁺-dependent disruption of the tight junctions and viral or bacterial infection of cell monolayers (15, 24, 28, 29). Although less is known about fence function compared with gate function, the distribution and expression of tight junctional proteins as well as the perijunctional actin may also regulate the fence function.

Compromises in intestinal epithelial gate and fence functions are common in both acute and chronically inflamed mucosa. In the latter, these changes are in large part due to specific downregulation of key tight junction-associated proteins such as occludin and ZO-1 by proinflammatory cytokines (13, 30, 42). However, in the context of acute (<24 h) mucosal inflammation, the mechanisms underlying rapid decreases in barrier function are less well defined.

In this study, we examined the effect and mechanism of action of reactive oxygen species (ROS), such as monochloramine (NH₂Cl), in acutely decreasing intestinal barrier function and compromising molecular fence function. NH₂Cl was selected for study because it is a physiologically relevant ROS produced in large quantity by inflammatory cell-derived hypo-
MATERIALS AND METHODS

Cell culture. All experiments were performed with Caco-2/bbe (C2) cells (31) (passages 53–60), a generous gift from Dr. Mark Mooseker (Yale University, New Haven, CT). C2 cells are clones from the Caco-2 cell line selected to express a well-developed brush border (31). C2 cells were grown in high-glucose DMEM supplemented with 10% (vol/vol) fetal bovine serum (FBS), 2 mM l-glutamine, 10 μg/ml transferrin, 50 U/ml penicillin, and 50 μg/ml streptomycin (all media components from GIBCO-Invitrogen; Grand Island, NY). Cells were kept at 37°C in 5% CO2, and medium was changed every 2–3 days. Cells were grown as confluent monolayers on collagen-coated permeable supports and were untreated (control) or treated with phalloidin, NH2Cl, and/or cytochalasin D and processed for globular (G-) and filamentous (F-)actin distribution. Cells were grown on collagen-coated Transwells (0.4-μm pore polycarbonate, Costar 3413; Corning, NY) except for confocal imaging, when cells were grown on collagen-coated polyethylene terephthalate filters (0.4-μm pore size, 4.2-cm² area, Falcon 3090). Confluent cultures of C2 cells were serum starved for 24 h before treatment with NH2Cl. NH2Cl was synthesized immediately before each use by reacting NH4Cl and NaOCl, which was quantitated at 242 nm (27). Vehicle controls were incubated with Tris-buffered saline with Tween 20 (T-TBS) containing 10 mM Tris (pH 7.4), 150 mM NaCl, and 5 mM KCl with 0.1% (vol/vol) Tween 20 with 5% (wt/vol) milk. Blots were incubated overnight at 4°C with specific primary antibodies [ZO-1, ZO-2, occludin, claudin-1, and claudin-5 (Zymed; San Francisco, CA)]. Blots were washed and incubated with species-appropriate peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch; West Grove, PA), washed with T-TBS, and developed using a chemiluminescence system (Super Signal, Pierce Chemical). Images were analyzed by densitometry using Hewlett-Packard precision scan (Hewlett-Packard scan jet precision3 5300) and Scion Image PC-Image J (Scion; Frederick, MD).

Determinations of filamentous and globular actin. C2 cell monolayers were treated with phalloidin, NH2Cl, and/or cytochalasin D and processed for globular (G-) and filamentous (F-)actin distribution. Cells were exposed to ice-cold H2O with 1 μM cytochalasin D and kept on ice for 1 h to depolymerize F-actin. Aliquots were removed for protein determination using the bicinchoninic acid procedure. Cells were solubilized with 3× Laemmli stop solution, and actin was analyzed in the fractions (20 μg protein/sample) by Western blot analysis by 12.5% SDS-PAGE using standard protocols and a rabbit polyclonal anti-actin serum (Cytoskeleton; Denver, CO).

Confocal indirect immunofluorescence. C2 cells were grown on collagen-coated permeable supports and were untreated (control) or exposed to either NH2Cl (0.6 mM) or phalloidin (100 μM) for 1 h before NH2Cl or cytochalasin D (2 μM) treatment on the apical and basolateral sides. After 1 h, cells were washed with K-PIPES buffer (80 mM, pH 6.5 with 1.5 mM CaCl2 and 1.5 mM MgCl2). With the use of a pH shift method to preserve cellular three-dimensional structure, fixation was performed using the same K-PIPES buffer described above but additionally containing 5 mM EDTA and 3.75% formaldehyde for 5 min at 37°C followed by Na-borate buffer (100 mM) with 3.75% formaldehyde (pH 11.0) for 10 min at room temperature. The fixed monolayers were washed with PBS containing 1.5 mM CaCl2 and MgCl2 (rinse buffer), permeabilized using rinse buffer with 0.1% (vol/vol) Triton X-100, and blocked using PBS containing 1% (wt/vol) BSA for 1 h. Cells were incubated overnight at 4°C with rabbit primary polyclonal antibody to ZO-1, ZO-2, claudin-1, or actin or mouse polyclonal antibody to claudin-5 in PBS containing 0.01% (vol/vol) Triton X-100. Cells were washed with rinse buffer with 0.01% (vol/vol) Triton X-100 and incubated in rinse buffer with 0.01% (vol/vol) Triton X-100 and then with Cy3-conju-
gated AffiPure goat anti-rabbit IgG or anti-mouse IgG (as necessary, Jackson Immunoresearch Laboratories; West Grove, PA). Cells were washed and mounted on glass slides using 38 μl of 5% (wt/vol) n-propyl-gallate (Sigma)-70% glycerol mounting solution. Localization of proteins was analyzed using a Fluoview 200 Laser Scanning Confocal Microscope equipped with a 543-nm argon laser at 60× magnification (zoom = ×2). Images were compiled as a maximum intensity projection from a Z series, which was 10 μm thick, and sections were taken every 0.2 μm. Images were analyzed and assembled using the unsharp mask and despeckle routines of Image J software. All images in a given set of experiments were obtained under identical imaging parameters.

Apical and basolateral membrane isolation. To determine whether NH2Cl alters the fence function of tight junctions, the apical membrane protein Na+/H+ exchanger isoform 3 (NHE3) and the basolateral protein Na+/K+ ATPase α-subunit were used. C2 cells were grown on permeable supports for 14 days and treated with NH2Cl (0.6 mM) for varying times. Cells were scraped off the filters in ice-cold PBS and pelleted (14,000 g for 20 s at 4°C). Cell pellets were lysed in 5 ml of ice-cold 10 mM Tris (pH 7.4) and 3 mM EDTA with a complete protease inhibitor cocktail and homogenized 20 strokes with a tight fitting Teflon pestle homogenizer. Unbroken cells, nuclei, and mitochondria were removed at 10,000 g for 5 min at 4°C, and 10 mM CaCl2 was added to the supernatant. This solution was kept on ice for 30 min, mixed every 5 min, and then spun at 10,000 g for 15 min at 4°C to obtain basolateral, endoplasmic reticulum (ER), and Golgi membranes. The supernatant was spun at 100,000 g for 30 min at 4°C to obtain apical membranes. The basolateral/ER/Golgi membrane pellet was resuspended in 1 ml of 40% Optiprep in the above buffer (Nycomed), and a 5–30% gradient of Optiprep in the buffer was layered on top. Gradients were spun at 37,000 rpm in a swinging bucket rotor (Sorvall; Newtown, CT) for 3 h at 4°C, and then 1-ml fractions were pulled and concentrated using 10-kDa molecular mass cutoff filters (Millipore; Milford, MA). Fractions were then analyzed for Na+/K+ ATPase activity using a colorimetric assay, and fractions that contained the greatest amounts pooled and analyzed by Western blot analysis for the apical membrane protein NHE3 as well as the α-subunit of Na+/K+ ATPase using 20 μg protein/sample with Western blot analysis of the apical membranes. The antibody to NHE3 has been characterized in our laboratory (11), and the antibody to the α-subunit of Na+/K+ ATPase was obtained from Upstate Biotechnology (clone 4644).

To determine the redistribution of the α-subunit of Na+/K+ ATPase using confocal microscopy, C2 cells on permeable supports were treated with 0.6 mM NH2Cl and fixed as described above after 60 min. When appropriate, cells were treated with phalloidin or cytochalasin D. Fixed cells were incubated overnight at 4°C with mouse monoclonal anti-α-subunit of ATPase (clone 4644 from Upstate Biotechnology) and then visualized using Cy2-conjugated anti-mouse IgG (Jackson Immunoresearch). Images were obtained as previously described except zoom = ×4 and a 488-nm argon laser was used. Both xy stack slices and xz reconstructions were deconvolved and then used to determine alterations in the distribution of this protein.

Immunochemistry of the human intestine. To assess membrane location of the α-subunit of Na+/K+ ATPase, paraffin sections of the human intestine were obtained from the pathology tissue bank of the Digestive Disease Research Core Center of The University of Chicago, a resource approved by the Institutional Research Board. Sections of the normal colon and colons with sporadic cancer, active Crohn’s disease, and active ulcerative colitis were stained with the murine monoclonal anti-α-subunit antibody described above using the Vector Elite ABC system (Burlingame, CA). After deparaffinization with xylene and hydration through ethanol-water, slides were briefly incubated in saline and then microwaved in the citrate buffer for 2 min followed by 3 min to cool for a total of three cycles. Endogenous peroxidase activity was quenched by incubation with 0.3% (vol/vol) hydrogen peroxide, and slides were then blocked with normal horse serum. Slides were next blocked with Avidin D blocking solution and then biotin blocking solution and incubated overnight with anti-ATPase antibody (1:500). Slides were then washed four times in saline and biotinylated anti-mouse antibody for 60 min. Slides were then washed four times in saline and for 30 min with Elite ABC complex followed by one wash in saline and then color developed using diaminobenzidine solution. Slides were stained with hematoxylin briefly after color development, and coverslips were mounted onto the slides using DPX medium (BDH Laboratories; Poole, UK).

Statistics. Results are means ± SE. Data comparisons were performed using Instat Software for the Mac (GraphPad; San Diego, CA) and generally using ANOVA using a Bonferroni correction for multiple comparisons.

RESULTS

Effects of actin-modulating agents on TER. As previously shown, the oxidant NH2Cl (0.6 mM) rapidly decreases TER of C2 cell monolayers (Fig. 1). To determine whether these effects were mediated by known oxidant-induced changes in the actin cytoskeleton and perijunctional ring, the effects of the actin stabilizer phalloidin (100 μM) on NH2Cl-induced changes in TER were investigated. As shown in Fig. 1, phalloidin alone had little effect on TER and did not inhibit the oxidant-induced decrease in TER despite preventing oxidant-induced disruption of the actin cytoskeleton (see Fig. 4). These results were surprising as the actin-disrupting agent cytochalasin D (2 μM; Fig. 1) rapidly decreased TER, implicating other potential sites or mechanisms of action for oxidant-induced compromise of epithelial barrier function.

To determine that the changes in TER reflected changes in paracellular permeability, apical to basolateral flux of the paracellular marker mannitol were performed. Similar to results with TER changes, NH2Cl and cytochalasin D rapidly increased mannitol permeability of the monolayers (Fig. 1). Phalloidin by itself had no effect on mannitol permeability, whereas phalloidin pretreatment before NH2Cl did not affect the changes in mannitol flux stimulated by NH2Cl (Fig. 1).

Alterations in tight junctional protein expression and distribution. Tight junctional barrier function can also be affected by changes in the distribution of specific junctional proteins or their levels of expression. The nonionic detergent NP-40 has been used to extract different “pools” of junctional proteins into NP-40-soluble and -insoluble fractions. For occludin, in particular, the level in the insoluble fraction correlates with changes in junctional permeability. Additionally, the degree of phosphorylation of occludin may also be important, as a highly phosphorylated form appears to comprise a large fraction of the insoluble or membrane fraction located within the tight junctional complex (38, 49).

NH2Cl rapidly stimulated decreased expression of ZO-1 and occludin (Fig. 2). In some experiments, increases in the NP-40-soluble forms of occludin were noted; however, only at times 15 min and earlier. Occludin has been noted to be degraded by a proteosomal pathway (45) and rapid degradation of occludin and ZO-1 may occur in C2 cells. This would make increased soluble levels of these proteins difficult to observe, particularly because the C2 cells already express substantial levels of both these proteins in the NP-40-soluble fraction. Notably, no changes in expression or distribution of claudin-1 or claudin-5 were observed with NH2Cl treatment. Changes in ZO-2 were modest and not consistently observed. Although not
the focus of the present studies, we observed a large fraction of claudins and occludin in C2 cells in the NP-40-soluble fraction. The role of this large cytoplasmic pool of occludin and claudins is unknown. Possibly, it serves as a reservoir that can be rapidly mobilized to the tight junctional membrane. Alternatively, this large pool may represent immature, incompletely processed precursors to mature protein. Of note, the total amount and ratio of the soluble to the insoluble pools of claudin-1 and claudin-5 remained constant during oxidant treatment of the cells over the time points examined (Fig. 2).

The effects of phalloidin and cytochalasin D, as an actin stabilizer and disruptor, respectively, on junctional protein expression and distribution were next determined. As shown in Fig. 3, NH$_2$Cl treatment caused a significant time-dependent decline in NP-40-insoluble ZO-1 and occludin levels, which was clearly evident by 30–45 min. This effect was blunted by pretreatment with phalloidin. Phalloidin alone had no effect. Cytochalasin D treatment caused a more modest and delayed decline in ZO-1 and occludin, in contrast to its pronounced effects on barrier function (compare Figs. 1 and 3).

As an adjunct approach for assessing the distribution of the junctional proteins, confocal imaging was used, which showed that NH$_2$Cl treatment of C2 cells resulted in disruption of perijunctional actin and beading of ZO-1 (Fig. 4). Cytochalasin D had a much more dramatic effect on actin, resulting in a clumped actin associated with dispersion of ZO-1, albeit to a lesser extent (Fig. 4). Phalloidin treatment attenuated many of the oxidant-induced effects on perijunctional actin and ZO-1. Although no NH$_2$Cl-induced changes in the soluble/insoluble distribution of claudin-1 and claudin-5 or ZO-2 were noted after Western blot analysis (Fig. 2), alterations in their cellular localization were observed by confocal imaging (Fig. 4). Under control (untreated) conditions, claudin-1 and claudin-5 are expressed on the cell periphery but also in the cytosol, particularly claudin-5. ZO-2 appeared to be found associated with actin as well as near the apical surface membrane. NH$_2$Cl stimulated dispersal of these proteins, particularly claudin-1 and ZO-2, throughout the cytosol, an effect observed to a lesser degree after actin disruption with cytochalasin D. Pretreatment of the cells with phalloidin before NH$_2$Cl prevented the dispersal of claudin-1 and claudin-5 as well as ZO-2. These data suggest important associative interactions of a number of tight junctional proteins with perijunctional actin that are dysregulated by oxidants.

**F/G actin distribution.** As another measure of actin dynamics, the ratio of F- to G-actin was determined. Cytochalasin D and NH$_2$Cl both stimulated actin depolymerization (Fig. 5). Phalloidin, by itself, did not appear to significantly affect F- and G-actin pools compared with untreated controls. However, phalloidin significantly prevented NH$_2$Cl-induced actin redistribution. Thus, despite the fact that the F-to-G-actin ratio was somewhat preserved when cells were pretreated with phalloidin before oxidant injury, TER decreased, suggesting that multiple targets or mechanisms might exist for oxidant-induced changes in barrier function.
Fence function of tight junctions. Tight junctions also play an important role in the maintenance of polarity, keeping transmembrane proteins of the basolateral and apical membranes separate, thereby preserving functional interactions essential for efficient epithelial cell physiology such as vectorial transport of nutrients and electrolytes. Fence function was assessed by following the distribution of the Na\(^+\)/H\(^+\) subunit of Na\(^+\)/K\(^+\)-ATPase between apical and basolateral membranes after cells were exposed to either cytochalasin D or NH\(_2\)Cl. Normally, this protein is predominantly localized to the basolateral membrane of polarized epithelium. As an additional determinant of fence function, distribution of the apical membrane transporter NHE3 was determined.

As shown in Fig. 6, NH\(_2\)Cl treatment of C2 cells was followed by a progressive redistribution of the basolateral α-subunit of Na\(^+\)/K\(^+\)-ATPase between apical and basolateral membranes after cells were exposed to either cytochalasin D or NH\(_2\)Cl. Normally, this protein is predominantly localized to the basolateral membrane of polarized epithelium. As an additional determinant of fence function, distribution of the apical membrane transporter NHE3 was determined.

As shown in Fig. 6, NH\(_2\)Cl treatment of C2 cells was followed by a progressive redistribution of the basolateral α-subunit of Na\(^+\)/K\(^+\)-ATPase, initially observed by 15 min. In contrast, the appearance of the Na\(^+\) pump α-subunit in the apical membrane after cytochalasin D treatment was minimal even at 30 min and was much less than that observed for NH\(_2\)Cl, even after 120 min (Fig. 6). It is notable that oxidant-

Fig. 3. Effects of NH\(_2\)Cl, phalloidin, and cytochalasin D on NP-40-insoluble levels of ZO-1 and occludin. C2 cell monolayers were treated with NH\(_2\)Cl without or with prior treatment (60 min) with phalloidin (100 μM) or solely with phalloidin or cytochalasin D (2 μg/ml) for varying times. NP-40-insoluble fractions were isolated as described in MATERIALS AND METHODS and analyzed for ZO-1 and occludin by Western blot analysis. Images shown are representative of 4 separate experiments. Densitometric analysis was performed by NIH Image 1.54 software and are means ± SE for n = 4. *P < 0.05 and +P < 0.01 compared with the time 0 untreated control by ANOVA employing a Bonferroni correction using Instat software.

Fig. 4. Enface confocal xy optical images of actin, ZO-1, ZO-2, claudin-1 (CLD-1), and CLD-5 in C2 cells. Images are of fluorescently labeled junction-associated proteins actin and ZO-1 after treatment of C2 cells with vehicle (control), cytochalasin D (2 μg/ml), phalloidin (100 μM), the oxidant NH\(_2\)Cl (0.6 mM) (each for 1 h), or phalloidin (100 μM) and then NH\(_2\)Cl (0.6 mM) (1-h phalloidin treatment before 1 h with NH\(_2\)Cl at the same concentrations as above).
induced changes in TER are more rapid than redistribution of the \( \alpha \)-subunit caused by both \( \text{NH}_2\text{Cl} \) and cytochalasin D. This would suggest that barrier function is more sensitive to alterations in certain key components of the tight junctional complex but that fence function is maintained by redundant systems or tight junctional proteins that must be collectively compromised before fence function is lost.

Confocal imaging was also performed to assess distribution of the \( \alpha \)-subunit of \( \text{Na}^+\text{-K}^+\text{-ATPase} \) after exposure to \( \text{NH}_2\text{Cl} \) and the actin destabilizer cytochalasin D. The images presented in Fig. 7, left, are en face (xy) images with the \( \alpha \)-subunit of \( \text{Na}^+\text{-K}^+\text{-ATPase} \) given the pseudocolor green. The red color present in some panels is transmitted reflected light used to monitor monolayer integrity. As can be observed, a large majority of the ATPase subunit appears at the outer plasma membrane (Fig. 7, left). To observe the \( \alpha \)-subunit on the lateral, basal, and apical aspects of the cells, \( \text{xy} \) axes were chosen so that the data was selected as shaving the sides of a cylindrical-shaped cell and is denoted by a line through the \( xy \) images. This ATPase is not localized to the apical pole in untreated monolayers; however, after \( \text{NH}_2\text{Cl} \) treatment, green staining appeared at the apical pole (Fig. 7, middle and right). There also appeared to be larger patches of green on what corresponds to the lateral membranes of the cells compared with control. This suggests that \( \text{NH}_2\text{Cl} \) might change the shape of cell to permit greater lateral visualization. Phalloidin treatment before \( \text{NH}_2\text{Cl} \) prevented some of the oxidant-stimulated apical redistribution, but the increase in lateral staining was blocked less by phalloidin pretreatment, potentially suggesting a role for actin stress fibers on lateral and basal poles of the cell. Additionally, cytochalasin D allowed some apical movement of the ATPase subunit, but notably more lateral staining was also observed.

To determine whether chronic inflammation in the human colon might affect membrane distribution of the \( \alpha \)-subunit of \( \text{Na}^+\text{-K}^+\text{-ATPase} \), paraffin sections from the normal human colon and colons with Crohn’s disease, ulcerative colitis, and colon cancer were examined. To better observe the staining, small sections in Fig. 8 are enlarged to the outside of each panel. In the normal colon, the \( \alpha \)-subunit of \( \text{Na}^+\text{-K}^+\text{-ATPase} \) was stained exclusively the basolateral membrane of the colonocytes (Fig. 8). Similarly, the \( \alpha \)-subunit of the \( \text{Na}^+\text{-K}^+\text{-ATPase} \) was exclusively found in the basolateral region membranes of colonocytes found in a section from a patient with colon cancer (sporadic cancer). However, in several regions of active inflammation found in sections from colons with Crohn’s disease or ulcerative colitis, the \( \alpha \)-subunit could be detected on the apical membrane, suggesting that the fence function of the tight junctions may be compromised in these human diseases.

Fig. 5. Effect of \( \text{NH}_2\text{Cl}, \) phalloidin, and cytochalasin D on the distribution of actin in filamentous (F) versus globular (G) pools. C2 monolayers were treated with \( \text{NH}_2\text{Cl} \) (0.6 mM) with or without phalloidin pretreatment (60 min, 100 \( \mu \text{M} \)), phalloidin alone, or cytochalasin D (2 \( \mu \text{g/ml} \)) alone, all for 60 min. Fractions containing F-actin and G-actin were isolated as described in MATERIALS AND METHODS, and actin levels in all fractions were analyzed by Western blot analysis. The percent actin in the F pool was determined by densitometric scanning of images. Data are means \( \pm \) SE for 3 separate experiments. * \( P < 0.05 \) and + \( P < 0.01 \) compared with the control by ANOVA using a Bonferroni correction.

Fig. 6. \( \text{NH}_2\text{Cl} \) stimulates rapid redistribution of a basolateral (Bl) membrane protein. C2 monolayers were treated with \( \text{NH}_2\text{Cl} \) (0.6 mM) with or without phalloidin pretreatment (60 min, 100 \( \mu \text{M} \)), phalloidin alone, or cytochalasin D (2 \( \mu \text{g/ml} \)) alone for varying times. Apical (Ap) and basolateral membrane fractions were analyzed by Western blot analysis for the apical membrane protein \( \text{Na}^+/\text{H}^+ \) exchanger isoform 3 (NHE3) and the basolateral membrane protein \( \alpha \)-subunit of \( \text{Na}^+\text{-K}^+\text{-ATPase} \). Images shown are representative of 3 separate experiments.
Fig. 7. Confocal micrographs of Cy2-tagged α-subunit of Na\(^+\)-K\(^+\)-ATPase in C2 cells. Cells were treated for 60 min with 0.6 mM \(\text{NH}_2\text{Cl}\) alone or after 1-h treatment with phalloidin (100 \(\mu\text{M}\)) or with cytochalasin D (2 \(\mu\text{M}\)). The red color is transmitted reflected light used to observe monolayer integrity. Apical slices are shown in the left. Vertical stack slices (\(xz\)) from regions marked with lines are shown in the middle and right, and a portion of this is enlarged (2×) to the right. White dashed lines have been included as representative \(xz\) reconstructions (\(n > 4\) from each panel) obtained from the series of \(xy\) slices (0.2 \(\mu\text{m}\) apart). Arrows are indicated on apical membranes of monolayers to indicate the apical movement of the α-subunit. All images have been deconvolved using the unsharpmask and despeckle routines in Image J.

DISCUSSION

ROS are major mediators of the inflammatory process and are produced in large quantity in inflammatory bowel diseases by infiltrating inflammatory cells and resident macrophages. Of the forms of ROS, chloramines, including \(\text{NH}_2\text{Cl}\), are physiologically relevant because of their rapid formation from immune cell-derived hypochlorous acid and amine-containing molecules, including gut flora-derived ammonia. Because it is long lived and cell permeant, \(\text{NH}_2\text{Cl}\) is highly cytotoxic, affecting multiple targets including membrane lipids, cellular proteins, and nucleic acids. Several studies have now shown that ROS may play a major role in compromised epithelial transport and barrier function associated with mucosal inflammation (1, 12, 20–21, 33–36, 44). This is particularly true under the stress conditions of acute inflammation leading to selective downregulation of barrier and transport-related protein expression (18, 48). This would suggest specific targeting of cellular targets of the apical barrier-related proteins of the
tight junction, such as ZO-1 and occludin, resulting in dysfunction.

A number of second messenger pathways may be involved in the oxidant-induced disruption of tight barrier function. Studies have shown that the xanthine/xanthine oxidase system (which generates predominantly superoxide) or acetaldehyde (which generates a number of reactive oxygen metabolites) (1) decrease monolayer TER (4, 33). The superoxide-induced TER decrease is blocked by the tyrosine kinase inhibitor genistein (35); however, the effects of oxidants on TER may also involve the regulation of phosphoprotein phosphatases such as protein tyrosine phosphatase, type 1B, which is inactivated under these conditions (36), and acetaldehyde, which nearly eliminates protein phosphatase, type 2B activity (1). Hydrogen peroxide stimulates the redistribution of occludin and ZO-1, an event that is dependent on the activation of c-Src kinase (4). Additionally, tyrosine phosphorylation of occludin may also influence its interaction with ZO-2 and ZO-3 (17). Oxidants decrease cellular levels of glutathione, which also regulates protein phosphatase activity (34) and could have additional effects on TER via other pathways that regulate tight junctional protein distribution and function.

The present results demonstrate that the physiologically relevant, long-lived, and cell-permeant oxidant \( \text{NH}_2 \text{Cl} \) causes alterations both in the cellular localization and levels of occludin and ZO-1. Concomitantly, \( \text{NH}_2 \text{Cl} \) stimulates disruption of actin filaments, including perijunctional actin (27), using concentrations that fall within the estimated range observed at sites of tissue inflammation (12). Gate and fence tight junctional functions may not be similarly regulated via modulation of ZO-1 and occludin distribution and expression and the state of actin. We used the actin filament stabilizer phalloidin to prevent the \( \text{NH}_2 \text{Cl} \)-induced depolymerization of actin. Although this paradigm was not effective in preventing \( \text{NH}_2 \text{Cl} \)-induced changes in TER, the redistribution and decreased expression of ZO-1 and occludin as well as movement of the basolateral \( \alpha \)-subunit of Na\(^+\)-K\(^+\)-ATPase were both largely inhibited by phalloidin pretreatment, suggesting a differential regulation of these events. We also used the actin depolymerizer cytochalasin D to address the roles of actin in regulating gate and fence functions. Whereas cytochalasin D stimulates rapid and large decreases of TER of equal magnitude to those with \( \text{NH}_2 \text{Cl} \), cytochalasin D stimulated slower and more modest changes in distribution and expression of ZO-1 and occludin and only after prolonged incubations was there movement of the ATPase \( \alpha \)-subunit to the apical membrane, an indication of altered cellular polarity. These results suggest complex regulation of both gate and fence function. Phalloidin was able to
prevent the NH$_2$Cl-stimulated redistribution of occludin and ZO-1 but was less able to prevent actin disruption by the oxidant and was not able to prevent NH$_2$Cl-stimulated changes in TER and mannitol flux. It appears that presence of ZO-1 and occludin in the NP-40-insoluble fraction, possibly at the tight junction, is not sufficient to maintain the gate function of the tight junctions. Cytochalasin D, which stimulates a rapid decrease in gate function but only a more modest and delayed change in fence function, might suggest that the perijunctional actin plays a modest role in the fence function. These hypotheses must be interpreted with caution as it is known that the perijunctional actin associates with ZO-1 and perhaps to other proteins of the tight junctional complex. The important prolonged maintenance of ZO-1 at the tight junction may be regulated not only by association with actin but also association with occludin and possibly other proteins of this multiprotein complex.

It should be noted that differences in gate and fence functions have been previously described (43). The actin depolymerizer mycocalide B increases paracellular permeability (gate function) with little change in fence function. Silencing RNA of occludin had no effect on the fence function of Madin-Darby canine kidney cells (type II) monolayers as assessed by BODIPY-labeled sphingomyelin in the apical membrane (50). Notable in these studies was that membrane cholesterol depletion, which decreases TER and stimulates actin cytoskeletal reorganization, was prevented in occludin-deficient cells due to a lack of occludin modulation of Rho-GTP activity. With regards to gate function in these studies, although no changes in basal TER were noted, permeability to monovalent organic anions increased (although no changes in Cl$^-$ or mannitol monolayer permeability were noted). Therefore, at present, both the formation and regulation of the fence as well as gate functions are incompletely understood. The roles of the integral membrane proteins occludin and claudins as well as the adherens junctions and a pivotal protein of this junction, JAM-1, are also hypothesized to play a role in the regulation of the fence function (8, 19). Alterations in fence function may not necessarily be seen for all plasma membrane proteins including the distribution of NHE. NHE3 was not affected by either cytochalasin D or NH$_2$Cl treatment. We speculate that some membrane proteins such as NHE3 are tethered by additional mechanisms or factors, which are oxidant-resistant and maintain polarity. Conversely, the apical membrane protein GP135 shows lateral mobility into the basolateral membrane after rotavirus infection (28). It would therefore appear that alterations in the fence function may have a profound effect on distribution or may have no effect at all.

In summary, this study provides important insights into the mechanisms of oxidant-mediated decreases in intestinal epithelial barrier function. Our data indicate that ROS target perijunctional actin and both the levels and distribution of key tight junction-associated proteins. Oxidant disruption of perijunctional actin is not by itself sufficient to cause these effects; rather, a combination of events mediates the oxidants actions. Additionally, oxidant-induced loss of barrier function is associated with compromised fence function. The latter can cause de-polymerization of critical membrane proteins, which is capable of severely disturbing many epithelial functions, particularly vectorial transport of nutrients and electrolytes. This prospect could contribute to development of malabsorption and diarrhea in patients with mucosal inflammation.

**REFERENCES**


Modulation of protein phosphorylation and dissociation of occludin-ZO-1 and E-cadherin-beta-catenin complexes from the cytoskeleton by oxidative stress. 
