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Remodeling of the tight junction during recovery from exposure to hydrogen peroxide in kidney epithelial cells

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Abstract

Renal ischemia-reperfusion injury results in oxidative stress-induced alterations in barrier function. Activation of the mitogen-activated protein (MAP) kinase pathway during recovery from oxidative stress may be an effector of oxidant-induced tight junction reorganization. We hypothesized that tight junction composition and barrier function would be perturbed during recovery from oxidative stress. We developed a model of short-term H_2O_2 exposure followed by recovery using Madin Darby Canine Kidney cells (MDCK II). H_2O_2 perturbs barrier function without a significant cytotoxic effect except in significant doses. ERK-1/2 and p38, both enzymes of the MAP kinase pathway, were activated within minutes of exposure to H_2O_2. Transient exposure to H_2O_2 produced a biphasic response in transepithelial electrical resistance (TER). An initial drop in TER at 6 hours was followed by a significant increase at 24 hours. Inhibition of ERK-1/2 activation attenuated the increase in TER observed at 24 hours. Expression of occludin initially decreased followed by partial recovery at 24 hours. In contrast, claudin-1 levels decreased and failed to recover at 24 hours. Claudin-2 levels markedly decreased at 24 hours; however, inhibition of ERK-1/2 activation was protective. Occludin and claudin-1 localization at the apical membrane on immunofluorescent images was fragmented at 6 hours after H_2O_2 exposure with subsequent recovery of appropriate localization by 24 hours. MDCK II cell recovery after H_2O_2 exposure is associated with functional and structural modification of the tight junction that are mediated in part by activation of the MAP kinase enzymes, ERK-1/2 and p38.

Keywords

Tight junction; kidney epithelium; oxidative stress; barrier function

Introduction

The tight junction encircles epithelial cells at the most apical portion of their lateral membranes, forming a barrier between cells and regulating paracellular transport across cell layers. The structure of the tight junction is dynamic and highly complex, consisting of multiple protein strands that join adjacent cell membranes and occupy the intercellular space. The integral

transmembrane tight junction proteins include occludin and the claudin family that fundamentally contribute to observed epithelial barrier properties along with the establishment of polarized epithelial membranes [1,2]. Tight junction disruption has been associated with barrier dysfunction in diseases that involve many different epithelial cell types [3,4], including as a unifying explanation for the multiple organ dysfunction syndrome seen in sepsis [5].

Normal renal physiology is characterized by controlled reabsorption and secretion of various solutes across renal tubular epithelium. Acute renal failure (ARF) is a common complication suffered by critically ill intensive care patients and often results in severe morbidity and/or mortality [6]. Ischemic ARF is characterized by sublethal and reversible tubular injury resulting in renal tubular dysfunction [4,7]. A growing body of evidence suggests that ischemia-reperfusion injury induced ARF is a disease of inflammation [8,9]. Oxidative stress-induced damage and activation of cell signaling cascades lead to the release of inflammatory mediators [10]. Changes at the tight junction induced by oxidative stress may contribute to aberrant ion and solute passage [11]. An association between renal ischemia and tight junction alterations has been demonstrated in multiple studies both *in vivo* and *in vitro*[4]. Despite its role in disease, the underlying mechanisms of tight junction injury and repair are still not well understood.

Common models of ARF *in vitro* include ATP depletion and exposure to nephrotoxic agents that mimic *in vivo* ischemic tubular injury. However, these models fail to fully explain all effects seen *in vivo* following ischemia-reperfusion injury [7]. This study addresses the effects of oxidative stress-induced injury to the tight junction in kidney epithelial cells with an emphasis on the reassembly of the tight junction during recovery. Here we present a model of recovery of the tight junction after exposure to the naturally occurring oxidant, hydrogen peroxide (H_2O_2). We hypothesized that tight junction reassembly and functional recovery following exposure to H_2O_2 would be mediated by mitogen activated protein (MAP) kinase activation.

Materials and Methods

Materials

Flasks, cell culture plates and Costar Transwell plates (Corning) were obtained from Fisher Scientific (Pittsburgh, PA). Hydrogen peroxide solution and fluorescein were purchased from Sigma-Aldrich (St. Louis, MO). U0126, SB202190, and cycloheximide were acquired from EMD Biosciences (San Diego, CA). The lactate dehydrogenase (LDH) activity cytotoxicity kit was purchased from Roche Applied Science (Indianapolis, IN). The caspase-3 assay kit, polyclonal rabbit anti-occludin, anti-claudin-1, and anti-claudin-2, and Alexa 488-conjugated anti-rabbit IgG antibodies were purchased from Invitrogen (Carlsbad, CA). Polyclonal rabbit anti-MAP Kinase 1/2 (ERK 1/2) was purchased from Upstate Cell Signaling Solutions (Lake Placid, NY). Polyclonal rabbit anti-p38 was purchased from Abcam (Cambridge, MA). Horseradish Peroxidase (HRP) conjugated anti-rabbit IgG antibody was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

Cell Culture

MDCK Type II (CCL-34) cells were obtained from ATCC (Manassas, VA) and grown in Dulbeco's Modification of Eagle's Medium (DMEM) (Mediatech, Herndon, VA), supplemented with 5% fetal bovine serum (FBS), ITS, penicillin (100 U/mL), and streptomycin (100 U/mL) in a humidified incubator at 37°C and 5% CO_2. For electrical resistance, flux measurements and immunofluoresent analysis cells were grown on 12-well Costar Transwell plates with 12 mm polycarbonate inserts with a 0.4 μm pore. For protein collection, LDH and Caspase-3 assays cells were grown on 24-well plates.

Experimental Conditions and Treatments

MDCK II cells were grown for 7–10 days to confluency (24-well plates) or until TER was stable for at least 2 days. At this time, cells were exposed to different concentrations of H_2O_2 (0.5, 3, and 10 mM) in complete DMEM for 60 minutes and then recovered with by changing to fresh media. TER and LDH release were measured and protein was collected at 2, 6, and 24 hours from the start of the exposure. Flux was measured at 6 and/or 24 hours from the start of exposure. Caspase-3 activity samples were collected at 2 and 6 hours after the start of exposure. For the MAP kinase enzyme activation experiments, MDCK II cells were changed to serum free DMEM for 1 hour to minimize serum-related phosphorylation; cells were then exposed to 3 mM H_2O_2, and protein was collected at various time points during the exposure. For the inhibitor experiments, cells were pre-incubated with the ERK-1/2 activation inhibitor U0126 (10 μM) or the p38 inhibitor SB202190 (10 μM) for 30 minutes prior to H_2O_2 exposure and fresh inhibitor was added with exposure and recovery media changes.

Determination of Cytotoxicity

LDH release into the media was used as a measure of cytotoxicity or plasma membrane integrity. MDCK II cells were changed to low serum (0.5% FBS) media at the start of treatment to avoid serum interference with the assay. At the indicated time points, media from the apical compartment was collected. An equal volume of assay substrate was added to each sample and absorbance was measured at 492 nm using a SpectraMax250 Platereader (Molecular Devices). A group of cells was lysed with 2% Triton-X 100 ten minutes prior to supernatant collection and used to determine total cellular LDH activity (high control). Untreated, control samples were used as low control. DMEM containing 0.5% FBS was used to determine background absorbance which was subtracted from each sample result. Cytotoxicity was calculated using the following formula: [(experimental sample absorbance − low control absorbance)/(high control absorbance − low control absorbance)] and expressed as percent cytotoxicity versus control.

Caspase-3 activity was used to assess cell death via apoptosis. At the appropriate time points after treatment, MDCK II cells were rinsed with sterile PBS and lysed for 30 minutes on ice. Cell lysate was collected and centrifuged for 5 minutes at 5000 rpm. Supernatant was collected and incubated for 30 minutes at room temperature with caspase-3 substrate. The fluorescence was measured (ex. 495nm and em. 520 nm) using a SpectraMax 250 fluorescence microplate reader (BioTech). Values were normalized for cell lysate protein concentration, as measured by a Spectrophotometer (Nanodrop, model ND-1000).

Measurement of Transepithelial Electrical Resistance (TER)

MDCK II cells were grown on Transwell plates until TER was stable for 2 days after a minimum of seven days in culture. Inserts were transferred aseptically to an Endohm Measurement Chamber and TER was directly measured using an Epithelial Voltohmmeter (World Precision Instruments, Model EVOMX). For testing, Transwells were kept in DMEM media and allowed to come to room temperature. The TER results are expressed as the measured resistance in Ohms multiplied by the surface area of the Transwell membrane.

Permeability Flux Assay

MDCK II cells were grown as described above for TER measurements. Permeability flux assays were performed by applying fluorescein (50 μM) to the apical chamber of Transwell inserts. Samples were collected from the basolateral (100 μL) and the apical (5 μL) compartments 60 minutes after fluorescein application. Apical samples were diluted with DMEM to a final volume of 100 μL. The fluorescent intensity in samples was measured (ex. 485nm and em. 528 nm) using a SpectraMax 250 fluorescence microplate reader (BioTech).

The diffusive flux rate (J_o) was calculated with corrections for volume, surface area, and time [12].

Western Blot Analysis

Analysis of protein expression via Western blot analysis was performed as previously described [13]. Protein concentration was determined using a Spectrophotometer (Nanodrop, model ND-1000). Equal concentrations of protein were gel-electrophoresed, followed by transfer to PDVF membranes. Membranes were probed for tight junction proteins or MAP kinase enzymes using a primary antibody concentration of 1:5,000 and a secondary antibody concentration of 1:10,000. The membranes were developed using the SuperSignal WestPico Chemiluminescence kit (Pierce, Rockford, IL) and imaged on a VersaDoc 3000. Bands were quantified with densitometry using the QuantityOne 1-D software (BioRad).

Immunofluorescent Analysis

Following treatment of MDCK II cells grown on Costar Transwells, cell layers were rinsed with sterile PBS on ice for ten minutes. Cells were permeabilized with an actin stabilizing buffer (0.2% Triton-X100, 100 mM KCl, 3 mM $MgCl_2$, 1.3 mM $CaCl_2$, 25 mM sucrose, and 2 mM HEPES, pH 7.1) for 2 min on ice, then fixed with cold 95% ethanol in PBS for 30 min. Cells were rinsed once with PBS and blocked with 5% BSA in PBS for 10 min. They were then incubated at room temperature for 1 hour with tight junction protein-specific primary antibodies [14]. Cells were rinsed three times with PBS and incubated at room temperature with anti-rabbit Alexa 488-conjugated secondary antibodies for 45 min. Primary (1:100) and secondary (1:200) antibodies were diluted in 1% BSA in PBS and centrifuged at 10,000×g for 15 min at 4°C prior to incubation. Following extensive rinse steps, the Transwell membranes were excised, coated with anti-fade medium, placed onto microscope slides, and stored in the dark until microscopic analysis using a Nikon 2000E microscope fitted with a z-stepper motor and MetaMorph Image Analysis Software.

Statistics

Data were analyzed with the StatPlus software package using either 1-way analysis of variance (ANOVA) followed by a protected Bonferroni Test for Differences Between Means or 2-way ANOVA followed by a protected Scheffe contrasts among pairs of means. A p value < 0.05 was considered significant, n indicates the number independent experiments. Errors are reported as SEM.

Results

A model of H_2O_2 exposure and recovery was developed

MDCK II cells grown on Transwell plates with stable TER (~100 Ω*cm^2) were exposed to various concentrations of H_2O_2 for 1 hour then recovered for 24 hours. There was no effect on TER during the 24 hour recovery after exposure to 0.5 mM H_2O_2. Recovery after both the 3 mM and 10 mM dose of H_2O_2 exposure resulted in a biphasic TER response. An initial decrease in TER occurred at 2 and 6 hours (40% decrease), followed by a marked increase at 24 hours after exposure (3–4 fold increase) (Fig 1A). Fluorescein flux rate measured at the 24 hour timepoint was not significantly perturbed by either the 0.5 mM or 3 mM H_2O_2 doses compared to control. However, a significant elevation in fluorescein flux was observed following exposure to 10 mM H_2O_2 (Figure 1B). Cytotoxicity, as measured by LDH release, was low and not significantly different from control except for at 24 hours after 10 mM H_2O_2 exposure (Figure 1C).

Inhibition of ERK-1/2 activation during recovery from H_2O_2 exposure enhances Caspase-3 activation

Inhibition of ERK activation with U0126 resulted in a significant and marked increase in caspase-3 activity at 6 hours when compared to vehicle control, antagonist alone and H_2O_2 exposed cells, thus demonstrating the importance of ERK activation in cell survival responses (Figure 2). Additionally, caspase-3 levels were significantly elevated over time in the H_2O_2 group at 6 hours when compared with the 2 hour measurement.

The biphasic functional response during recovery is dependent on new protein synthesis

In order to examine the requirement for new protein synthesis during junctional remodeling, the protein synthesis inhibitor cycloheximide (CHX, 10 μM) was employed. MDCK II cells exposed to 3 mM H_2O_2 in the presence of CHX for the entire protocol resulted in a significant decrease in TER at 6 and 24 hours in contrast to the marked increase in TER at 24 hours seen with H_2O_2 alone. In contrast, CHX addition at the 6 hour interval after H_2O_2 exposure resulted in TER returning to control levels at the 24 hour time point (Figure 3). Addition of CHX alone did not alter TER over the 24 hour period (data not shown).

Tight junction protein expression changes in response to H_2O_2 exposure and recovery

Exposure to 3 mM H_2O_2 followed by recovery resulted in decreased occludin protein levels at 2 and 6 hours (both 70% of control levels) with a relative increase towards baseline levels at 24 hours (Figure 4A). Interestingly, a higher molecular weight occludin band was observed at the 2-hour interval consistent with previously studies indicating occludin was highly phosphorylated [15]. Addition of the ERK antagonist was occludin protective at the 6-hour interval but had no effect on expression at 24 hours since occludin levels had recovered. Claudin-1 levels were decreased at 2 hours with a further and sustained depression at 6 hours and 24 hours (Figure 4B). ERK inhibition did not influence the claudin-1 response to H_2O_2. Claudin-2 levels were markedly decreased at 24 hours after exposure to H_2O_2 (Figure 4C). ERK inhibition was claudin-2 protective at the 24-hour interval. β-actin (Figure 4D) levels were not significantly altered by the given treatments.

Tight junction protein localization changes in response to H_2O_2 exposure and recovery

Tight junction protein localization was determined using indirect immunofluorescence (Figure 5). MDCK II cells in the control condition displayed the expected chicken-wire tight junction protein localization pattern. Exposure to 3 mM H_2O_2 followed by recovery resulted in a substantial delocalization of the tight junction proteins occludin and claudin-1 at 6 hours. By 24 hours after exposure, occludin localization had returned to near-normal, while claudin-1 localization was recovering but appeared delayed in comparison to the occludin response which is consistent with the expression data. Claudin-2 localization appears not to be markedly impacted by H_2O_2 exposure however an increase in membrane ruffling is observed at 24 hours (Figure 5).

The MAP kinase enzymes ERK-1/2 and p38 are activated by H_2O_2 exposure

Phosphorylated (activated) ERK-1/2 levels are typically low in MDCK II cells. Exposure to 3 mM H_2O_2 resulted in rapid and time-dependent manner increase in phosphorylated ERK-1/2 for up to 60 minutes of exposure (Figure 6A). Phosphorylated (activated) p38 levels were robustly elevated over time with exposure to 3 mM H_2O_2; similarly, there were low-levels of phosphorlylated p38 in control cells (Figure 6B). We have confirmed that the ERK-1/2 activation inhibitor U0126 (10 μM) completely blocks detection of phosphorylated ERK-1/2. The p38 inhibitor, SB202190 (10 μM) attenuates phosphorylated p38 in our cell system. However, it should be noted that the SB202190 does not block the activity of the upstream kinase but is rather a specific antagonist of p38 activity including inhibition of

autophosphorylation [16, 17]. Additionally, we have demonstrated that U0126 partially attenuates p38 phosphorylation, but SB202190 does not interfere with ERK-1/2 phosphorylation (Figure 6C).

Inhibition of ERK-1/2 activation and p38 activity alters the functional changes seen during recovery from H_2O_2 exposure

Inhibiting ERK activation significantly attenuated the increase in TER seen at 24 hours following H_2O_2 exposure. In contrast, inhibiting p38 activity with SB202190 potentiated the TER response when compared to the H_2O_2 exposure alone (Figure 7A). MAP kinase antagonist (U0126 or SB202190) treatment alone had no effect on TER over the same time period (data not shown). Paracellular flux rates at the 6 hour time point were elevated in all the H_2O_2 exposed groups, and SB202190 potentiated the increase flux when compared to the H_2O_2 exposure alone. Flux rates at the 24 hour time point in the H_2O_2 exposed groups were similar to control levels (Figure 7B).

Discussion

Redox signaling by reactive oxygen species, including H_2O_2, has been demonstrated in many cell types during normal cell functions, as well as pathologic disease states [18–20]. ERK activation is known to exert pleiotropic effects on cellular systems including participation in regulating responses to H_2O_2 exposure. Cell type and H_2O_2 exposure parameters influence the responses, along with a complex system of differential activity by protein tyrosine phosphatases and kinases by the oxidation of well-conserved catalytic cysteine residues [18, 21]. This study was designed to examine the relationship between MAP kinase activation and tight junction dynamics during recovery from oxidative stress in MDCK II cells. In addition to the kidney, our results have important clinical relevance to other organ systems such as the brain and lung where the existence of various tight junction proteins have been recently described and oxidative stress is known to be an important cause of injury in both adults and the developing human [22–24]

Flux is a dynamic measure of solute permeability influenced by tight junction strand remodeling and cytoskeletal events that reflects a different picture of junctional function than TER [25]. A previous study in MDCK II cells demonstrated a decrease in TER in response to H_2O_2 exposure, which was associated with tight junction protein disorganization. Reassembly of the junction during recovery was shown to be dependent on tyrosine phosphorylation [26]. In the current study, we demonstrate that TER has a biphasic response to H_2O_2 exposure and recovery. The initial decrease correlates with elevated paracellular flux findings, consistent with increased permeability of the junction to water and solutes, including ions. The substantial elevation in TER at the later interval suggests an altered tight junction composition with significantly decreased ionic permeability. Paracellular flux is initially elevated during the first phase of recovery period but then returns to normal levels at later intervals suggesting a structurally disrupted tight junction that recovers, which is consistent with the immunofluorescence findings. Further investigation into the mechanisms involved with recovery of TER and cellular barrier function, including MAP kinase regulation and altered expression of various key tight junction proteins, may potentially lead to more effective therapeutic strategies to prevent or limit cellular injury. For example, such future experiments would provide valuable insights into understanding the role of tight junctions in blood-brain barrier injury following hypoxic-ischemic insult given that little is currently understood in this area [27].

An area of interest is ascertaining whether ERK activation is beneficial or detrimental to cell survival [28]. For instance, constitutively active MEK, an upstream activator of ERK, has been shown to provide resistance to H_2O_2 exposure [29]. In contrast, a recent study using renal

proximal tubular cells showed that ERK activation due to H_2O_2 exposure is associated with increased mitochondrial permeability and necrosis [30]. We find that inhibition of ERK activation during exposure to H_2O_2 and the subsequent recovery interval significantly elevated caspase-3 activity when compared to caspase-3 activity in the H_2O_2-group. This suggests that ERK activity during an acute oxidative stress event is anti-apoptotic in MDCK II cells.

In the present study, we examined the role of protein synthesis on the TER response during recovery from H_2O_2 exposure by employing cycloheximide treatment. We found that protein synthesis was required for recovery from H_2O_2 exposure. Interestingly, the marked elevation in TER during the later phase of recovery was dependent on *de novo* protein synthesis. In contrast to our findings, Meyer *et. al.* suggested that tight junction recovery was not dependent on new protein synthesis, as evidenced by no change in the recovery patterns with cycloheximide treatment [26]. Of note, they used both a shorter H_2O_2 exposure time (30 minutes) and recovery period (8 hours) in their experiments, which at least in part may have accounted for the difference in results.

We report that occludin expression and localization is dynamically regulated in response to H_2O_2 exposure and recovery. Occludin was the first transmembrane protein described as a component of the tight junction [31] and alterations in occludin in initial studies appeared to affect tight junction function. However, occludin −/− mice develop structurally and functionally normal tight junctions [32], and there is now considerable evidence that the claudin super-family of tight junction proteins confer the tissue-specific differences in resistance and charge-selectivity [33]. Although the contribution of occludin to barrier function remains to be further elucidated, occludin does also appear to be involved in the cell-signaling function of the tight junction [33–35]. As such, it is interesting that in our model of recovery from oxidative stress, occludin expression and localization is initially disrupted, but then recovers by 24 hours. Although these changes in expression and localization may not be responsible for the demonstrated functional changes, it is possible that these changes in occludin play a role in transmission of the cell-signaling events induced by H_2O_2 exposure. Basuroy *et. al.* showed that H_2O_2–mediated barrier dysfunction in an intestinal epithelial cell line is in part secondary to the rapid phosphorylation of occludin. The study also demonstrated that epidermal growth factor prevents H_2O_2–mediated barrier dysfunction in what appears to be an ERK-1/2 mediated pathway. In addition, phospho-ERK appeared to be co-localized with occludin, providing evidence that ERK-1/2 plays a role in the regulation of tight junction proteins [36]. Consistent with an occludin phosphorylation event [15] our study showed a higher mobility occludin band during the early phase of recovery and ERK inhibition was occludin protective. A study in MDCK cells transfected with mutant occludin demonstrated a similar uncoupling of flux and TER. However, this effect occurred in an opposite manner, as occludin overexpression caused an increase in flux, but no apparent change in TER [25]. In accordance with previous studies the changes in occludin expression and localization that occur during recovery from H_2O_2 exposure likely contribute to the uncoupling of the flux and TER responses during the later phases of recovery.

Activation of ERK-1/2 is integral to the regulation of paracellular permeability in MDCK cells [37,38], and ERK-1/2 regulation of H_2O_2-mediated tight junction permeability has been established in multiple cell types [36,39,40]. Consistent with these previous studies, we have demonstrated that ERK-1/2 is rapidly activated upon H_2O_2 exposure. Lipschutz *et. al.* demonstrated that transfection of MDCK Type II cells with activated ERK-1/2 causes a decrease in claudin-2 levels and an increase in TER [38], which is consistent with our results. Claudin-2 creates cation-specific pores in the tight junction generating a low resistance phenotype, TER in is inversely related to claudin-2 levels in MDCK cells [41,42]. The phenomenon of elevated TER during the recovery phase after exposure to H_2O_2 has not previously been reported. We propose that *de novo* protein expression in addition to ERK-1/2

activation is required to lower claudin-2 levels generating the marked changes in observed TER.

The initial identification of p38 MAP kinase demonstrated it was tyrosine phosphorylated in response to bacterial endotoxin and hyperosmolarity [43]. In an independent investigation it was also established that SB202190 and other similar compounds blocked cytokine production by inhibiting cytokine-suppressive antiinflammatory drug-binding protein now known as p38 [44]. The p38 family plays a central role in the response to a variety of stressors and is an essential component of numerous cellular signaling cascades [45]. Focusing on p38 and barrier function it has been demonstrated that p38 was activated in endothelial cells in response to H_2O_2 and inhibition of p38 activity was barrier protective [46]. In Caco-2 cells a model of colonic epithelium, H_2O_2 activated p38 resulting in a compromised barrier function, both inhibitors of p38 or overexpression of a p38 repressor (Wip1) was junction protective [47]. Activation of p38 following H_2O_2 exposure was necessary for EGF receptor activation and beta-catenin phosphorylation in renal proximal tubular cells. The p38 activity resulted in a cellular dediferentiation that is essential for tissue repair and remodeling during renal injury [48,49]. In the current study, we demonstrate rapid phosphorylation of p38 following H_2O_2 exposure. Inhibition of p38 activity using SB202190 resulted in a significant elevation of paracellular flux during the first phase of recovery when compared to the H_2O_2-group alone. Interestingly, p38 inhibition potentiated the TER response at 24 hours in contrast to ERK inhibition.

In summary, we report a novel model of recovery from exposure to oxidative stress in a kidney epithelial cell line. The model presented demonstrates that MAP kinase activation alters both the structure and function of the tight junction following exposure to H_2O_2, which is a naturally occurring oxidant that has been implicated in oxidative stress-induced damage to renal tubular epithelium during ischemic ARF. Dynamic changes in occludin, claudin-1 and claudin-2 expression and localization contribute to the functional changes observed during recovery from H_2O_2 exposure. Importantly, reassembly of the junction that occurs during recovery results in a modified tight junction composition produced lasting functional consequences, which potentially alters the physiology of the tissue. This study adds to the growing body of evidence that injury of the tight junction in renal tubular epithelial cells contributes to the pathophysiology of ARF and that remodeling and repair of tight junction proteins is important for subsequent clinical recovery of renal function. In addition, future investigation into the role of tight junction proteins in injury and repair in other organ systems to include the brain, gastrointestinal tract and lung following insults such as oxidative stress will be important and may ultimately lead to more effective clinical therapies,

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Abbreviations

JAM	junctional adhesion molecule
ARF	acute renal failure
LDH	lactate dehydrogenase
TER	transepithelial electrical resistance
H_2O_2	hydrogen peroxide

MAP mitogen activated protein

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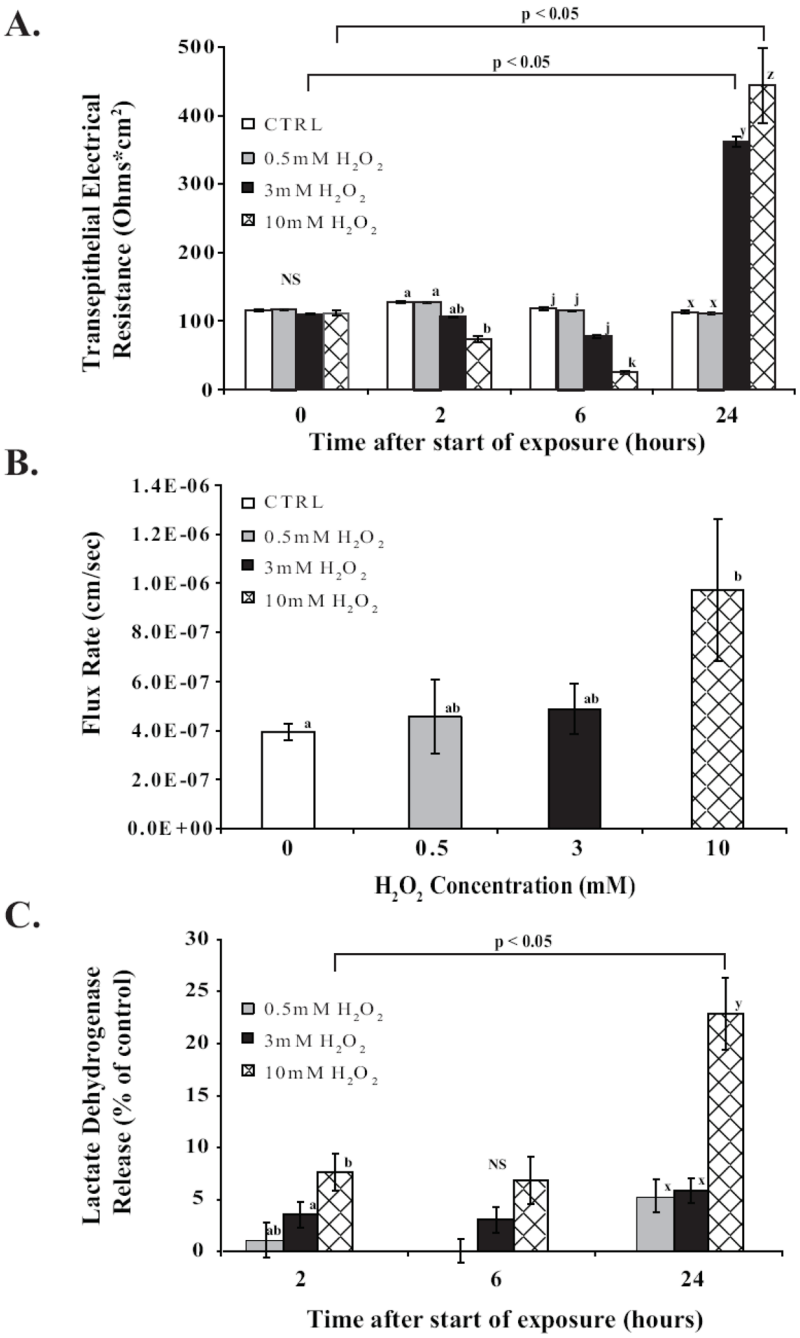

Figure 1.

Functional changes in MDCK II cells during recovery from H_2O_2 exposure. CRTL indicates MDCK II cells cultured under identical conditions that were not exposed to H_2O_2. Transepithelial Electrical Resistance (TER) response was measured in MDCK cells during recovery from H_2O_2 exposure at four intervals **(Panel A)**. Error bars represent SEM, n=6 at all time points. Letters denote significant differences within groups at designated times, brackets indicate significant differences across times; 2-way ANOVA followed by Scheffe post-hoc test, $p<0.05$. Paracellular flux rate determination 24 hours after H_2O_2 exposure **(Panel B)**. Fluorescein was added 24 hours after exposure. Samples were collected 60 minutes later from apical and basal chambers and analyzed. Error bars represent SEM, n=12 for 0 and 3 mM

H_2O_2 and n=6 for 0.5 and 10 mM H_2O_2. Letters denote significant differences within groups, 1-way ANOVA followed by the Bonferroni test, $p<0.05$. Cytotoxicity as measured by LDH release from MDCK II cells during recovery from H_2O_2 exposure **(Panel C)**. Samples of media from the apical chamber were collected before exposure and at 2, 6, and 24 hours after the start of exposure, and then assayed for LDH release. Error bars represent SEM, n=6 at all time points. Letters denote significant differences within groups at designated times, brackets indicate significant differences across times; 2-way ANOVA followed by Scheffe post-hoc test, $p<0.05$.

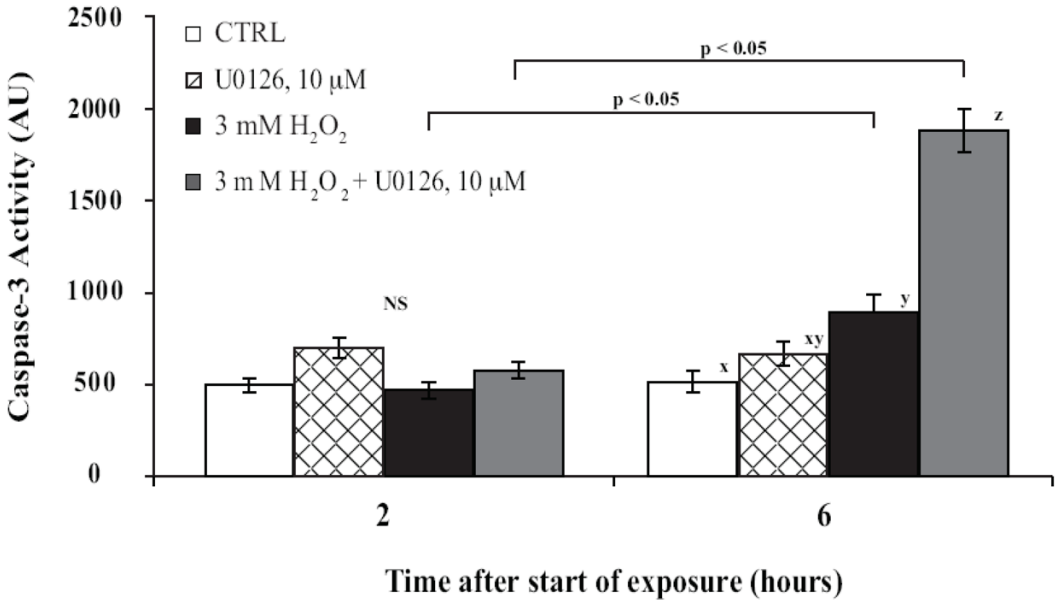

Figure 2.

ERK inhibition potentiates caspase-3 activity during recovery from H_2O_2 exposure. Cells were grown in 24-well culture plates and exposed to 3mM H_2O_2 for 60 minutes, then recovered for up to 6 hours. U0126 (10μM) was added 30 minutes prior to H_2O_2 exposure and maintained throughout exposure and recovery. Error bars represent SEM, n=6 at all time points. Letters denote significant differences within groups at designated times, brackets indicate significant differences across times; 2-way ANOVA followed by Scheffe post-hoc test, $p<0.05$.

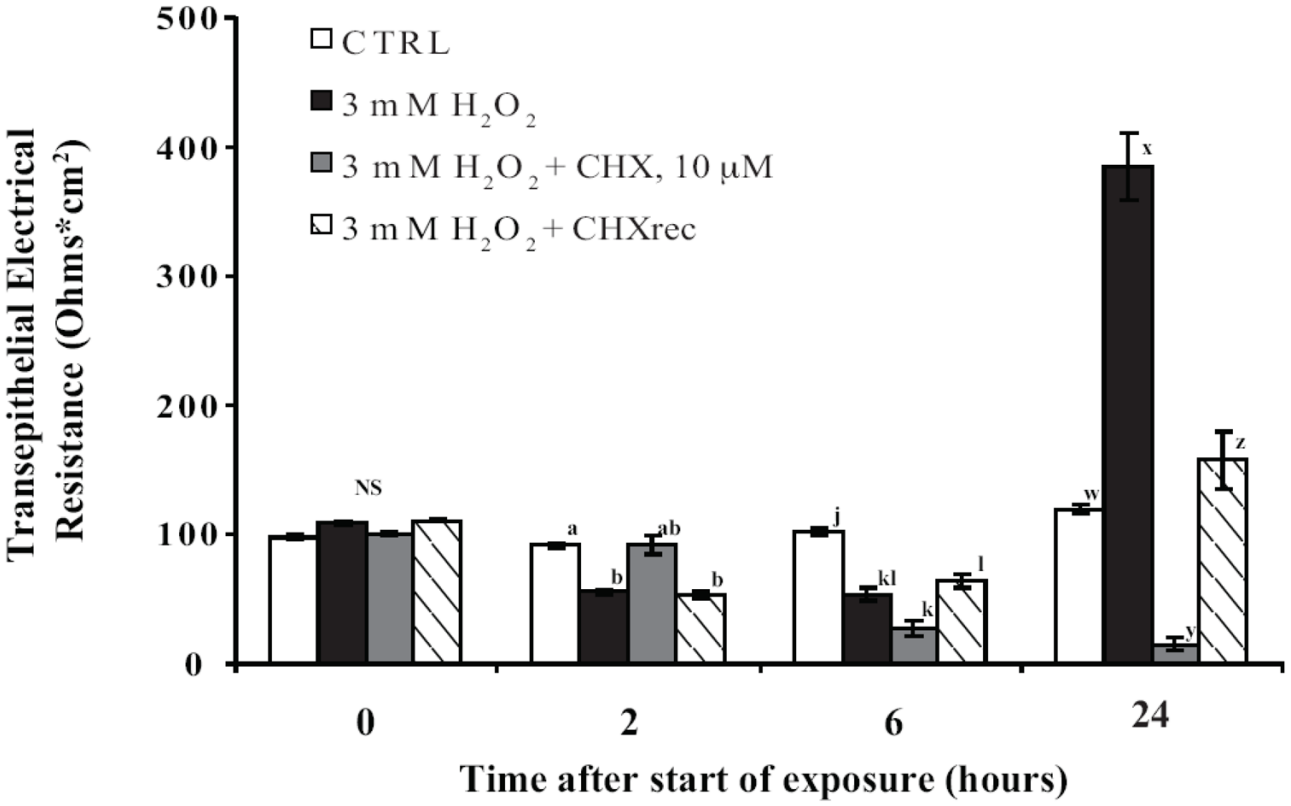

Figure 3.

Effects of cycloheximide (CHX) on functional recovery after H_2O_2 exposure. Cells were grown in 12-well transwells and exposed 3 mM H_2O_2 for 60 minutes then recovered for 24 hours. CHX (10 μM) was added either with exposure or at 6 hours after the start of exposure (CHXrec). TER was measured before exposure and at 2, 6, and 24 hours following the start of exposure. Error bars represent SEM, n=6 at all time points.

Letters denote significant differences within groups at designated times, 2-way ANOVA followed by Scheffe post-hoc test, $p<0.05$.

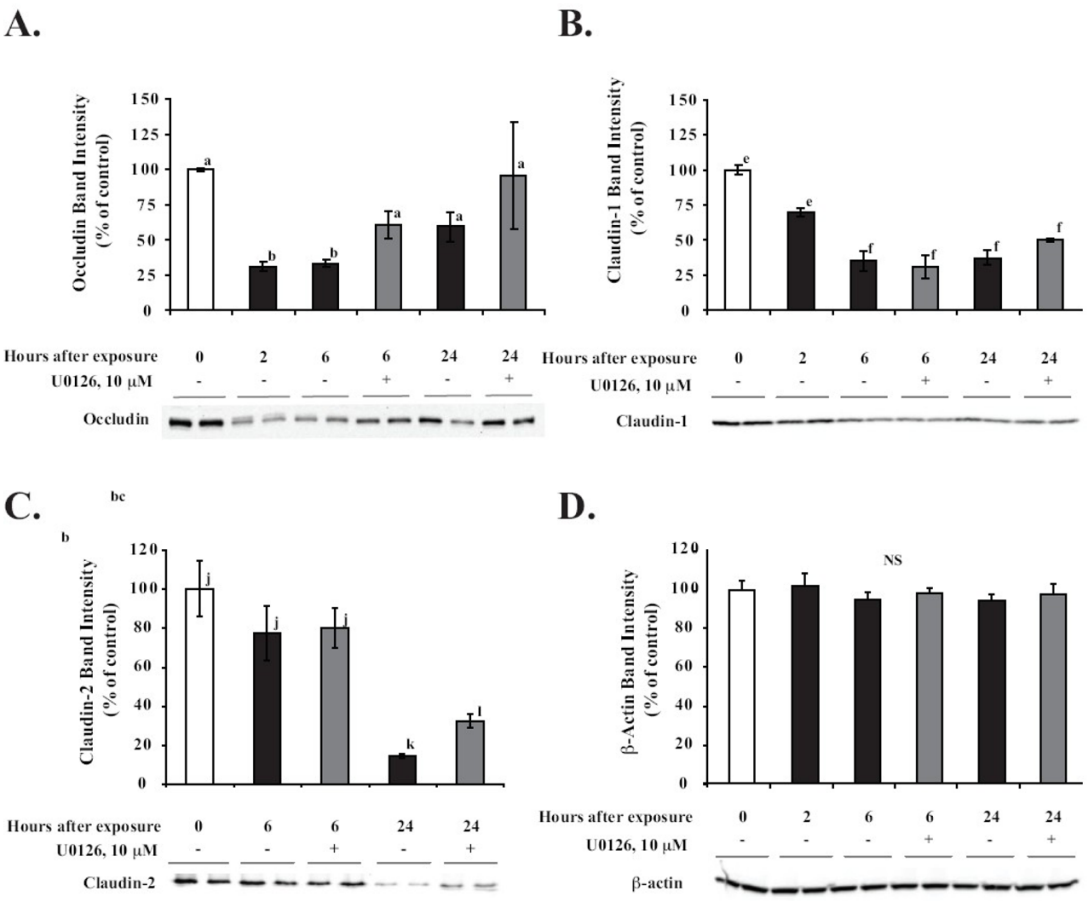

Figure 4.

Expression of Tight Junction proteins during recovery from H_2O_2 exposure. Cells were exposed to 3 mM H_2O_2 for 60 minutes then recovered. Total protein was collected at indicated time points. Panel (**A**) is densitometric analysis of the occludin content with a representative Western blot for occludin below. Panel (**B**) is densitometric analysis of the claudin-1 content with a representative Western blot for claudin-1 below. Panel (**C**) is densitometric analysis of the claudin-2 content with a representative Western blot for claudin-2 below. Panel (**D**) is densitometric analysis of the β-actin content with a representative Western blot for β-actin below. For each densitometry time point, n=4 and error bars represent SEM. Letters denote significant differences within groups, 1-way ANOVA followed by the Bonferroni test, $p<0.05$.

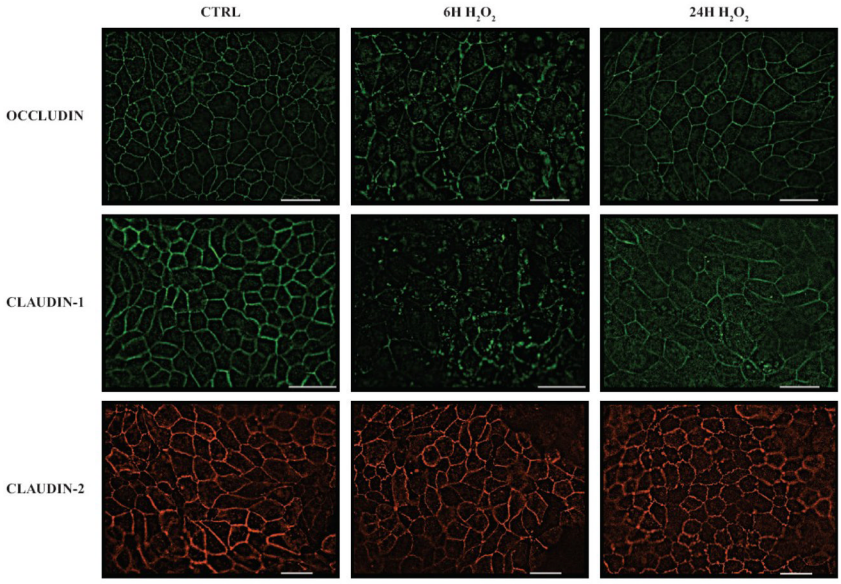

Figure 5.

Indirect immunofluorescence of occludin, claudin-1, and claudin-2 localization. MDCK II cells grown on Transwells were exposed to 3mM H_2O_2 for 60 minutes, then recovered for 6 and 24 hours. A. Cells were then fixed, permeabilized, and probed for occludin, claudin-1 or claudin-2. Fluorescence microscopy images (60X) were obtained using a Nikon Eclipse TE-2000U microscope. Bar=20μm

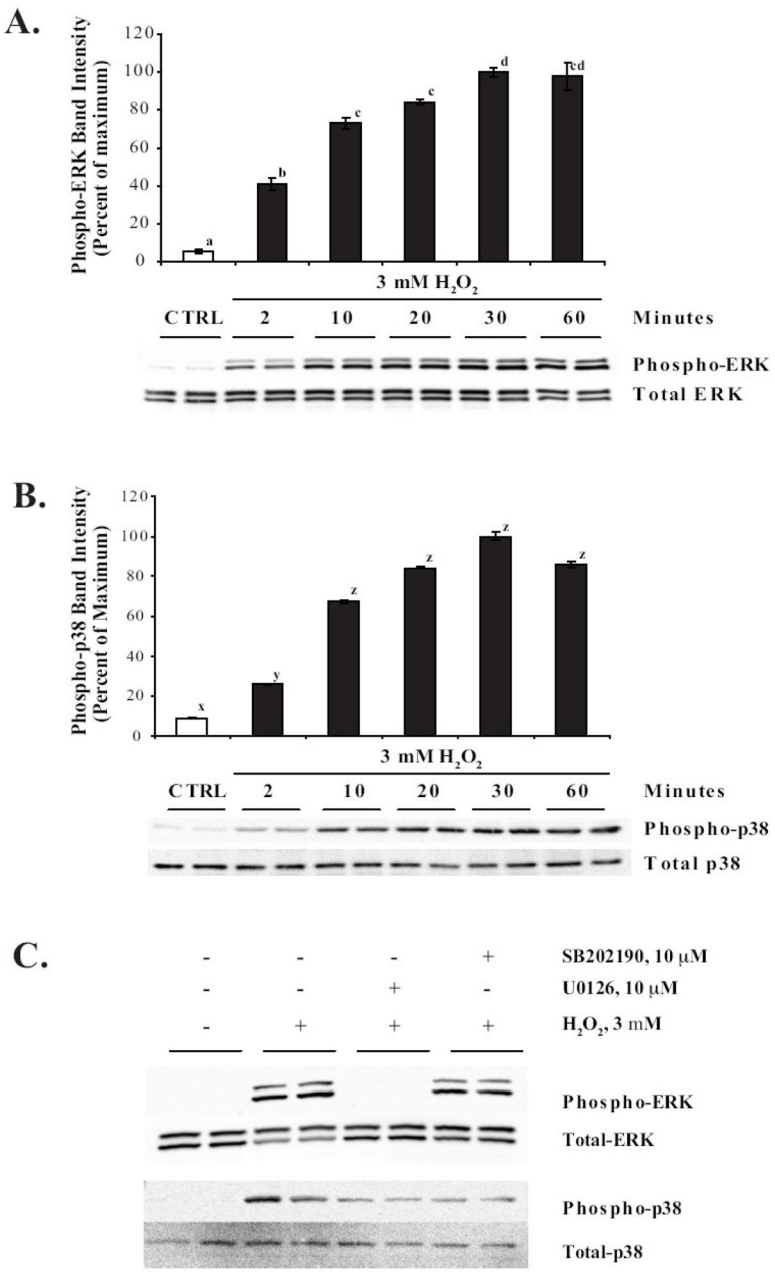

Figure 6.

H_2O_2 exposure rapidly activates the MAP kinase enzymes ERK-1/2 and p38. Confluent MDCK II cells were exposed to 3mM H_2O_2 in serum free media, and total protein was collected at indicated time points. Panel (**A**) is densitometric analysis of the phospho-ERK content with a representative Western blot for phospho- and total ERK below. Panel (**B**) is densitometric analysis of the phospho-p38 content with a representative Western blot for phospho- and total p38 below. For each densitometry time point, n=4 and error bars represent SEM. Letters denote significant differences within groups, 1-way ANOVA followed by the Bonferroni test, $p<0.05$. Panel (**C**) examines ERK-1/2 and p38 phosphorylation in the presence of specific MAP kinase inhibitors. Confluent MDCK II cells were exposed to 3 mM H_2O_2 in serum free media for 20 minutes and total protein was collected. U0126 (10 μM) and SB202190 (10 μM) were added

15 minutes prior to start of H_2O_2 exposure. Samples are representative duplicates for the Western blot.

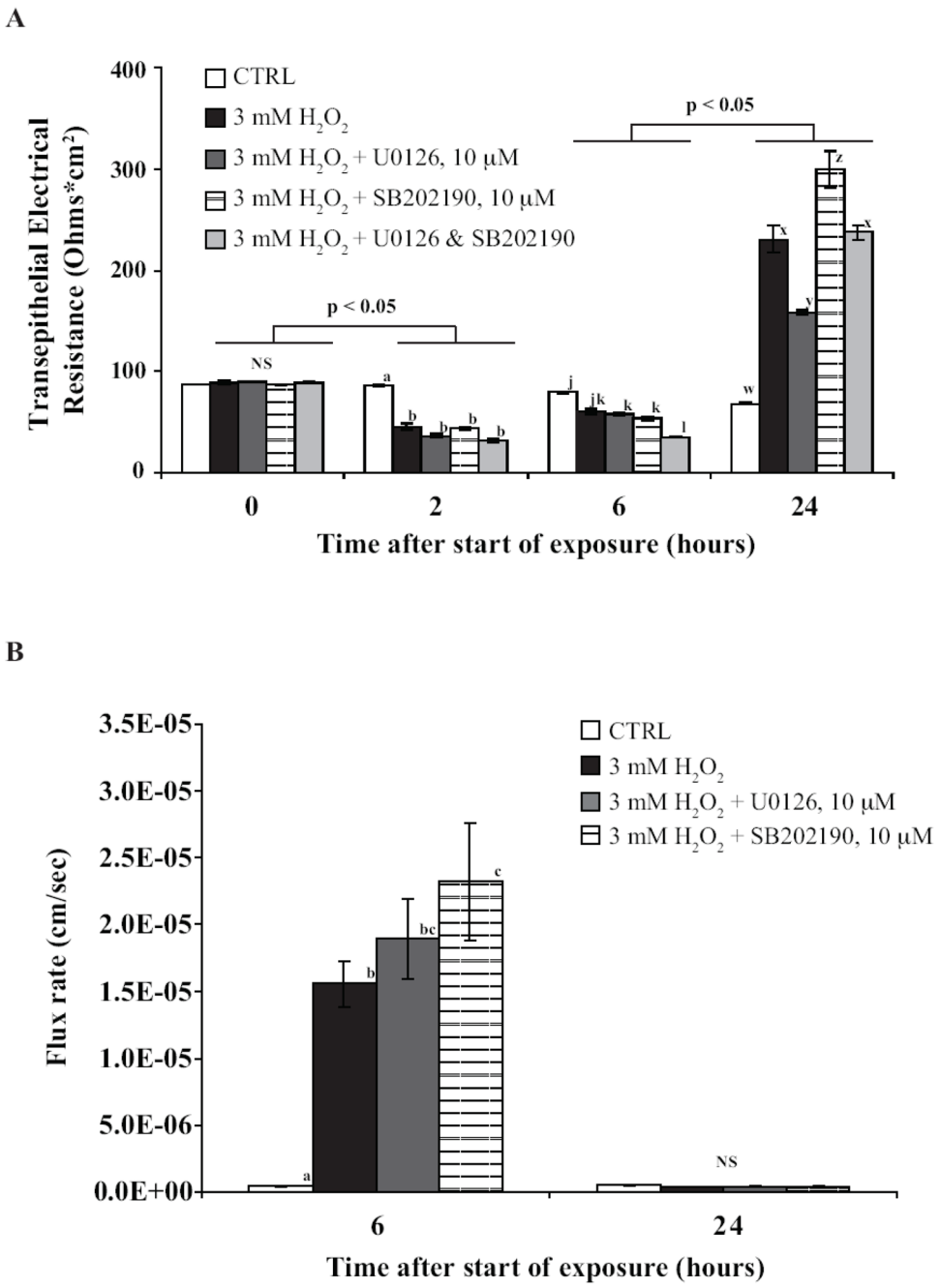

Figure 7.

Functional changes in MDCK II cells are altered by the ERK inhibitor U0126 and p38 inhibitor SB202190 during recovery from H_2O_2 exposure. Cells were grown in 12-well transwells and exposed to 3mM H_2O_2 for 60 minutes, then recovered for 24 hours. Inhibitors (both 10μM) were added 30 minutes prior to H_2O_2 exposure and maintained throughout exposure and recovery. TER was measured before start of exposure and at 2, 6, and 24 hours following the start of exposure as seen in panel **(Panel A)**. Paracellular flux rates were measured at 6 and 24 hours following the start of exposure **(Panel B)**. Error bars represent SEM, n=6 at all time points. Letters denote significant differences within groups at designated times, brackets

indicate significant differences across times; 2-way ANOVA followed by Scheffe post-hoc test, $p<0.05$.