Role of Phosphatidylinositol 3-Kinase in Oxidative Stress-Induced Disruption of Tight Junctions

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Sheth et al

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**Abbreviations:** PI 3-kinase, phosphatidylinositol 3-kinase; PBS, phosphate buffered saline; HRP, horse radish peroxidase, SDS, sodium dodecyl sulfate; PMSF, phenylmethyl sulfonyl fluoride; TLC, thin layer chromatography; ZO-1, ZO-2 & ZO-3, zonula occludens 1, 2 & 3; XO+X, xanthine oxidase + xanthine; TER, transepithelial electrical resistance, ACS, actin cytoskeleton, MCS, membrane cytoskeleton, TS, Triton-soluble fraction.
ABSTRACT

A recent study suggested that PI 3-kinase may interact with occludin, however, there exists no evidence of its role in the regulation of tight junctions. Activation of PI 3-kinase by oxidative stress, and its role in disruption of tight junctions was examined in Caco-2 cell monolayer. The oxidative stress-induced decrease in electrical resistance, increase in inulin permeability, and redistribution of occludin and ZO-1 were reduced by a PI 3-kinase inhibitor, LY294002. Oxidative stress-induced tyrosine phosphorylation and dissociation from the actin cytoskeleton of occludin and ZO-1 were reduced by LY294002. The regulatory subunit of PI 3-kinase, p85, and the PI 3-kinase activity were co-immunoprecipitated with occludin, which were rapidly increased by oxidative stress. Oxidative stress resulted in increased translocation of p85 from the intracellular compartment into the intercellular junctions. Pair wise GST-pull down assay showed that GST-occludin (C-terminal tail) binds to recombinant p85. This study shows that oxidative stress increases the association of PI 3-kinase with the occludin, and that PI 3-kinase activity is involved in oxidative stress-induced disruption of tight junction.
INTRODUCTION

Epithelial tight junctions form a barrier to the movement of pathogens, toxins and allergens from the intestinal lumen into the tissue. The disruption of tight junctions plays an important role in the pathogenesis of a number of gastrointestinal diseases (1, 2). Studies reported during the past decade show that the tight junctions are composed of at least three types of transmembrane proteins, occludin (3), claudins (4) and junctional adhesion molecule (5). Transmembrane proteins interact with other intracellular plaque proteins such as ZO-1, ZO-2, ZO-3, cingulin and 7H6, which anchor the transmembrane proteins to the actin cytoskeleton (6-9). Growing evidence indicates that the activities of intracellular signaling molecules regulate tight junctions. Studies indicate that signaling pathways involving protein kinases, G-proteins, Rho/Rac GTPases regulate the tight junction permeability (10-20). Tyrosine kinases such as c-Yes, c-Src and focal adhesion kinase (FAK) are localized at the vicinity of tight junctions and adherens junctions (3). A recent study demonstrated that c-Src plays an important role in the disruption of tight junctions (21), while c-Yes may be involved in the assembly of tight junction (22). Another study, by in vitro bait peptide pull down assay, showed that the regulatory subunit of PI 3-kinase, p85, binds to the intracellular C-terminal region of occludin (23), raising the possibility of the role of PI 3-kinase-dependent signaling pathway in the regulation of tight junctions. A recent study showed that PI 3-kinase inhibitors prevented VEGF-induced Ser/Thr-phosphorylation and redistribution of occludin and ZO-1 in bovine aortic endothelial cells (24). However, association of the PI 3-kinase with the tight junction and its role in regulation of epithelial permeability is not known.
A significant body of evidence indicates that oxidative stress disrupts epithelial tight junctions and increases the paracellular permeability in Caco-2 cell monolayer (16-20). Oxidative stress-induced paracellular permeability was inhibited by tyrosine kinase inhibitors, and was associated with tyrosine phosphorylation of a wide spectrum of proteins, including occludin, ZO-1, E-cadherin and β-catenin (17-19). In a recent study it was demonstrated that the expression of kinase-inactive c-Src mutant delays the oxidative stress-induced disruption of tight junctions in Caco-2 cell monolayers (21) indicating the important role of c-Src in regulation of tight junction.

In the present study we examined the role of PI 3-kinase in the oxidative stress-induced disruption of tight junctions in Caco-2 cell monolayers. Results show that: 1) Inhibitor of PI 3-kinase activity reduces the oxidative stress-induced disruption of tight junctions, 2) oxidative stress increases the association of PI 3-kinase with occludin, and induces translocation of p85 to the intercellular junction, and 3) occludin interacts directly with p85. This study for the first time shows that PI 3-kinase is associated with occludin in Caco-2 cell monolayers, and plays an important role in the regulation of epithelial tight junctions by oxidative stress.
MATERIALS AND METHODS

Chemicals: Cell culture reagents and supplies were purchased from GIBCO-BRL (Grand Island, NY). FITC-inulin, vanadate, SDS, xanthine oxidase, xanthine, genistein, protease inhibitors, streptavidin agarose, protein-A Sepharose and protein-G Sepharose were purchased from Sigma Chemical Company (St Louis, MO). LY294002 was from Calbiochem (San Diego, CA). Phosphatidylinositol was purchased from Avanti Polar Lipids (Alabaster, AL), and $^{32}$P-$\gamma$-ATP was from ICN radiochemicals (Irvine, CA). All other chemicals were of analytical grade purchased either from Sigma Chemical Company or Fisher Scientific (Tustin, CA).

Antibodies: Mouse monoclonal anti-p85, recombinant HRP-conjugated anti-p-Tyr, biotin–conjugated anti-p-Tyr, anti-mouse IgG and HRP-conjugated anti-rabbit IgG antibodies were purchased from Transduction Laboratories (Lexington, KY). Rat monoclonal anti-ZO-1 antibody was purchased from Chemicon International Inc. (Temecula, CA). Mouse monoclonal anti-occludin, rabbit polyclonal anti-ZO-1, HRP-conjugated anti-occludin, Cy3-conjugated anti-rabbit IgG, AlexaFluor 488-conjugated anti-mouse IgG and Oregon Green-conjugated anti-rat IgG antibodies were from Molecular Probes (Eugene, OR).

Cell culture Caco-2 and MDCK cells purchased from American Type cell collection (Rockville, MD) were grown under standard cell culture conditions as described before (20). Cells were grown on polycarbonate membranes in Transwells (6.5 mm, 12 mm or 24 mm; Costar, Cambridge, MA), and experiments conducted on 11-13 days (6.5 or 12 mm Transwells) or 17-19 days (24 mm Transwell) after seeding.
Treatment with oxidative stress: Oxidative stress was induced as previously described (20). Briefly, cell monolayers were incubated in PBS (Dulbecco’s saline containing 1.2 mM CaCl₂, 1 mM MgCl₂ and 0.6% BSA) in the absence or presence of a mixture of xanthine oxidase (20 mU/ml) and xanthine (0.25 mM) (XO+X) with or without LY294002 (25 µM). Control cell monolayers were incubated in PBS without XO+X and inhibitors.

Measurement of transepithelial electrical resistance (TER): TER was measured as described previously (16) using a Millicell-ERS Electrical Resistance System (Millipore, Bedford, MA). TER calculated as Ohms.cm² by multiplying it with the surface area of the monolayer. The resistance of the polycarbonate membrane in Transwells (approximately 30 Ohms.cm²) was subtracted from all readings.

Unidirectional flux of inulin: Transwells with the cell monolayers were incubated under different experimental conditions in the presence of FITC-inulin (0.5 mg/ml) in the basal well. At different times after XO+X treatment, 100 µl each of apical and basal media were withdrawn, and fluorescence measured using a fluorescence plate reader (BioTEK Instruments, Winooski, Vermont). The flux into the apical well was calculated as the percent of total fluorescence administered into the basal well per hour per cm² surface area.

Immunofluorescence Microscopy: After treatment with XO+X in the absence or presence of LY294002 for varying times Caco-2 cell monolayers (12 mm) were washed in PBS and fixed in acetone: methanol (1:1) at 0°C for 5 min. Cell monolayers were blocked in 3% non-fat milk in TBST (20 mM Tris, pH 7.2, and 150 mM NaCl) and incubated for one hour with primary antibodies, rabbit polyclonal anti-occludin, mouse
monoclonal anti-p85, and rat monoclonal anti-ZO-1 antibodies, followed by incubation for one hour with secondary antibodies, Oregon green-conjugated anti-rat IgG, AlexaFluor 488-conjugated anti-mouse IgG and Cy3-conjugated anti-rabbit IgG antibodies. The fluorescence examined by a confocal laser scanning microscopy (Biorad MRC1024), and images from Z-series sections (1 µm) collected by using comos (confocal microscope operating system). Images were stacked using the software, Confocal Assistant 4.02, and processed by Adobe Photoshop (Adobe Systems Inc., San Jose, CA).

**Preparation of cytoskeletal fractions:** Cell monolayers in Transwell (24 mm) were washed twice with ice-cold PBS, and incubated for 5 min with lysis buffer-CS (Tris buffer containing 1.0% Triton-X100, 2 µg/ml leupeptin, 10 µg/ml aprotinin, 10 µg/ml bestatin, 10 µg/ml pepstatin-A, 1 mM vanadate and 1 mM PMSF). Cell lysates were centrifuged at low speed 15,600 x g for 4 min at 4°C to sediment the high-density actin cytoskeleton. The pellet was suspended in 200 µl of lysis buffer-CS. Protein contents in different fractions were measured by BCA method (Pierce Biotechnology Inc (Rockford, IL). Cytoskeletal and Triton-soluble fractions were mixed with equal volume of Laemmli’s sample buffer (2x concentrated) and heated at 100°C for 5 min.

**Immunoprecipitation:** After XO+X treatment for varying times Caco-2 cell monolayers (24 mm) were washed with ice-cold 20 mM Tris (pH 7.4) and actin cytoskeleton and Triton-soluble fraction were prepared. Actin suspension was sonicated for 10 seconds in lysis buffer-CS. Actin lysate and Triton-soluble fraction (1.0 mg protein/ml) were incubated with 2 µg of anti-occludin antibodies at 4°C for 16 hr.
Immune complexes were isolated by precipitation using protein-A Sepharose (for 1 h at 4°C). Washed beads were suspended in 20 ml of assay buffer to measure PI 3-kinase activity, or heated in Laemmli’s sample buffer for immunoblot analysis.

For tyrosine phosphorylation studies, cytoskeletal fractions were extracted in lysis buffer D (0.3% SDS in 10 mM Tris buffer, pH 7.4, containing 1 mM vanadate and 0.33 mM PMSF) by heating at 100°C for 5 min. For co-immunoprecipitation of PI 3-kinase with occludin, cytoskeletal fractions were extracted in lysis buffer N at 4°C for 30 min. Cytoskeletal extracts were incubated overnight at 4°C with 2 µg of biotin-conjugated anti-p-Tyr or 2 µg of rabbit polyclonal anti-occludin antibodies. Immunoprecipitation was carried out overnight as described above. Immune complexes were precipitated by incubation for one hour with streptavidin-Agarose or protein-A Sepharose at 4°C. Anti-p-Tyr immune complexes were immunoblotted for occludin and ZO-1. Anti-occludin immunoprecipitates were immunoblotted for occludin and p85 or used for PI 3-kinase assay.

**Immunoblot analysis:** Proteins were separated by SDS-polyacrylamide gel (4-12 % gradient) electrophoresis and transferred to PVDF membranes. Membranes blotted for occludin, ZO-1 and p85 by using specific antibodies in combination with HRP-conjugated anti-mouse IgG or HRP-conjugated anti-rabbit IgG antibodies. The blot was developed using ECL chemiluminescence method (Amersham, Arlington Heights, IL).

**PI 3-kinase assay:** PI 3-kinase assay was carried out as described by Avanti Polar Lipids (Alabaster, AL). The occludin immune complexes were incubated in a 50 ml assay system consisting of 25 mM MOPS buffer, pH 7.0, 5 mM MgCl₂, 1 mM EGTA, 1
mM sodium orthovanadate, 40 μg of phosphatidylinositol substrate (presonicated) and 150 μM ATP containing 10 μCi $^{32}$P-$\gamma$-ATP. Reaction mixture was incubated at 37°C for 30 min. Reaction stopped by the addition of two volumes of 6 M HCl in methanol. Lipids were extracted with chloroform and then separated by thin layer chromatography on calcium depleted, activated silica gel 60 (Whatman, Maidstone, England) using water:n-propanol:acetic acid (34:65:1, v/v/v) solvent system. TLC plates were exposed to X-Ray films to determine the level of incorporation of $^{32}$P to substrate.

**Preparation of GST-C-occludin and GST-p85:** C-terminal tail of chicken occludin as a GST fusion protein, GST-C-occludin, and GST-p85 were prepared in E. coli DH5α cells and purified using GSH-Agarose as described before (5). cDNA for C-terminal tail of occludin (amino acids 354-503) in pGEX vector was a gift from Dr. J.M. Anderson and A. Fanning, University of North Carolina, and the cDNA for p85 in pGEX vector was kindly provided by Dr. Marcello Arsura (Department of Pharmacology, University of Tennessee, Memphis).

Pairwise binding assay: This particular assay detects binding between two individually purified proteins. For this purpose, we generated occludin and p85 as GST-fusion proteins (e.g., GST-Occludin C-tail; a.a 354 to 503 and GST-p85; a.a.1-724. The GST portion of GST-p85 was clipped off with thrombin. Binding assays were performed using GST-C-Occludin and thrombin-cleaved p85, and excess glutathione-Sepharose beads was used to ‘pull down’ the bound complex. Complexes are then immunoblotted for p85. This assay determines direct interaction between two proteins.
RESULTS

PI 3-kinase activity is required for the oxidative stress-induced disruption of tight junctions.

To determine the role of PI 3-kinase in the regulation of tight junctions, the effect of the LY294002, a PI 3-kinase inhibitor, on oxidative stress-induced disruption of tight junctions was evaluated. Treatment of cell monolayers with XO+X resulted in a time-dependent decrease in TER (Fig. 1A) and increase in inulin permeability (Fig. 1B). Pretreatment of cell monolayers with LY294002 significantly reduced the XO+X-induced decrease in TER and increase in inulin permeability. The effect of LY294002 on XO+X-induced changes in TER and inulin permeability was concentration-dependent (Fig. 1C & D). Immunofluorescence confocal microscopy showed that XO+X induced a redistribution of occludin and ZO-1 from the intercellular junctions (Fig. 2), which was prevented by pretreatment of cell monolayers with LY294002.

PI 3-kinase inhibitor prevents oxidative stress-induced tyrosine phosphorylation of occludin and ZO-1, and their dissociation from the actin cytoskeleton.

Previous studies showed that oxidative stress-induced disruption of tight junctions in Caco-2 cell monolayers was associated with tyrosine phosphorylation of occludin and ZO-1, and their release from the actin cytoskeleton (20). Therefore, the present study evaluated the effect of LY294002 on XO+X-induced changes in tyrosine phosphorylation and cytoskeletal association of tight junction proteins. XO+X treatment resulted in an increase in tyrosine phosphorylation of occludin and ZO-1 in the actin cytoskeleton, the membrane cytoskeleton and the Triton-soluble fractions. LY294002 reduced XO+X-
induced tyrosine phosphorylation of occludin and ZO-1 in all fractions (Fig. 3A). XO+X also induced a time-dependent reduction in the amounts of occludin and ZO-1 associated with the actin cytoskeleton, which was accompanied by an increase in occludin and ZO-1 in the Triton-soluble fraction (Fig. 3B). LY294002 reduced the XO+X-induced decrease in occludin and ZO-1 in the actin cytoskeleton and their increase in Triton-soluble fractions. LY294002 by itself produced no significant effect on cytoskeletal association or tyrosine phosphorylation of occludin and ZO-1 (data not shown).

**Oxidative stress induces a rapid activation of PI 3-kinase.**

The above studies indicate that PI 3-kinase activity is required for the oxidative stress-induced disruption of tight junction. Therefore, we evaluated the effect of XO+X on the localization of the regulatory subunit of PI 3-kinase and PI 3-kinase activity in the cytoskeletal fractions. XO+X induced a rapid increase in the amount of PI 3-kinase regulatory subunit associated with the actin cytoskeleton and the membrane cytoskeleton, while it was reduced in Triton-soluble fractions (Fig. 4A & B). XO+X also increased PI 3-kinase activity in the actin cytoskeleton with the maximal increase achieved at 15 min (Fig. 4C & D). Activity in Triton-soluble fraction was slightly reduced at later time points.

**Oxidative stress increases association of PI 3-kinase with the occludin.**

A previous study raised the possibility of association of PI 3-kinase with the tight junction (23). To determine the interaction of PI 3-kinase with the tight junction complex in Caco-2 cell monolayers we evaluated co-immunoprecipitation of PI 3-kinase with occludin. Considerable amount of p85, the regulatory subunit of PI 3-kinase, was co-immunoprecipitated with occludin precipitated from the actin cytoskeleton (Fig. 5A &
B). The level of p85 co-immunoprecipitated with occludin was rapidly increased by XO+X treatment. Low levels of PI 3-kinase activity were detected in the immune complexes of occludin prepared from the resting epithelial actin cytoskeleton and Triton-soluble fraction (Fig. 5C & D). Treatment with XO+X rapidly increased PI 3-kinase activity associated with the immune complexes of occludin prepared from both the actin cytoskeleton and the Triton-soluble fraction. Only trace amounts of PI 3-kinase activity were detected in the immune complexes of occludin prepared from the membrane cytoskeleton (data not shown). PI 3-kinase activity was dramatically low in immune complexes of occludin prepared from the cells treated with XO+X after pretreatment with LY294002 (Fig. 5E & F).

To determine the translocation of PI 3-kinase to the vicinity of tight junction we analyzed the effect of oxidative stress on localization of p85 by immunofluorescence confocal microscopy. P85 was predominantly localized in the intracellular compartments at the apical part of the cell (Fig. 6). Treatment with XO+X induced a minor change in the localization of p85 at 5 min, but at 30 and 60 min after treatment p85 was predominantly localized at the intercellular junctions, and co-localized with the occludin (Fig. 6).

**Direct binding of p85 with occludin**

In order to determine the direct interaction of C-terminal tail of occludin with p85 we generated recombinant GST-fused C-occludin (C-terminal 150 aminoacids) and GST-p85. GST-p85 was cleaved with thrombin, and p85 was incubated with varying concentrations of GST-C-
Occludin. GST pull down assay showed that recombinant p85 binds C-occludin in a concentration-related manner (Fig. 7). GST alone showed only a minimal binding to p85.

**Oxidative stress increases paracellular permeability in MDCK cell monolayer by a PI 3-kinase-dependent mechanism**

In order to demonstrate the PI 3-Kinase-mediated regulation of tight junction in another epithelial model we evaluated the effect of LY294002 on oxidative stress-induced increase in permeability in MDCK cell monolayers. Incubation of MDCK cell monolayers with XO+X decreased TER (Fig. 8A) and increased inulin flux (Fig. 8B) in a time-dependent manner. This increase in paracellular permeability was associated with reorganization of occludin and ZO-1 from the intercellular junctions (Fig. 8C). Pretreatment of cell monolayers with LY294002 significantly reduced XO+X-induced decrease in TER, increase in inulin permeability and reorganization of occludin and ZO-1 (Fig. 8).
DISCUSSION

Oxidative stress disrupts tight junction- and adherens junction-based cell-cell adhesion in Caco-2 cell monolayer by a tyrosine kinase dependent mechanism (20). The oxidative stress-induced disruption of the tight junction and the adherens junction is associated with the tyrosine phosphorylation of occludin, ZO-1, E-cadherin and β-catenin. Recent studies demonstrated that oxidative stress induces a rapid activation of c-Src, and that c-Src activity is required for the oxidative stress-induced disruption of the tight junction (21). The present study shows that oxidative stress increases the level of PI 3-kinase associated with the occludin, and that PI 3-kinase activity mediates the oxidative stress-induced disruption of tight junctions in Caco-2 cell monolayers. This is the first evidence for the association of PI 3-kinase with a tight junction protein, and for the role of PI 3-kinase activity in regulation of epithelial tight junctions.

A significant reduction of oxidative stress-induced decrease in TER, increase in inulin permeability and redistribution of occludin and ZO-1 by LY294002 indicate that PI 3-kinase activity plays an important role in the oxidative stress-induced disruption of tight junction in Caco-2 cell monolayer. Previous studies demonstrated that occludin, ZO-1, E-cadherin and β-catenin undergo rapid phosphorylation on tyrosine residues during the oxidative stress-induced disruption of tight junctions (20) and that tyrosine phosphorylation reduces the interactions between occludin and ZO-1, ZO-2 and ZO-3 (25). The present study shows that LY294002 reduces oxidative stress-induced tyrosine phosphorylation of occludin and ZO-1. Previous studies also demonstrated that occludin and ZO-1 bound to actin cytoskeleton correlates well with the barrier function of the epithelium (20). Disruption of tight junctions by oxidative stress reduces the amounts of
occludin and ZO-1 bound to the actin cytoskeleton, which was prevented by genistein, a tyrosine kinase inhibitor. The present study shows that LY294002 also reduces the oxidative stress-induced dissociation of occludin and ZO-1 from the actin cytoskeleton.

Therefore, PI 3-kinase activity is required for the oxidative stress-induced tyrosine phosphorylation and dissociation from the actin cytoskeleton of tight junction proteins, and therefore for the disruption of tight junctions. However, the temporal relationship between oxidative stress effect on tyrosine phosphorylation, release from the actin cytoskeleton and disruption of tight junction is not clear. Significant increase in tyrosine phosphorylation and release of occludin from the actin cytoskeleton occurred by 60 min, while less than 20% of TER was reduced. It is likely that mechanisms downstream to activation of PI 3-kinase and c-Src, and other lateral mechanisms are involved in oxidative stress-induced disruption of TJ.

The fast migrating occludin bands that appeared in oxidative stress-treated cells (Fig. 3) are likely result of proteolytic degradation of occludin. In our previous study (20) we showed that oxidative stress induces proteolytic degradation of occludin, and the low MW degradation products are predominantly present in MCS and TS fractions. The metalloproteinase inhibitor, 1,10-phenanthroline, significantly reduced the oxidative stress-induced degradation of occludin. However, this inhibitor did not prevent oxidative stress-induced disruption of TJ, and therefore occludin degradation was considered uninvolved in oxidative stress-induced disruption of TJ and increase in permeability. Additionally, genistein prevented oxidative stress-induced increase in permeability without an effect on occludin degradation. Similarly, the present study shows that LY294002 reduces
oxidative stress-induced tyrosine phosphorylation of occludin (Fig. 3A) with no effect on proteolytic degradation of occludin (Fig. 3B).

The requirement of PI 3-kinase activity in the regulation of tight junction, and the previous in vitro study that raised the possibility of interaction between the regulatory subunit of PI 3-kinase and the C-terminal sequence of occludin (23) suggest that PI 3-kinase may be associated with the tight junction complex in Caco-2 cells, and that oxidative stress may alter this interaction. The present study shows that the regulatory subunit of PI 3-kinase and the PI 3-kinase activity are associated with the immune complexes of occludin prepared from the actin cytoskeleton of the resting epithelium. Oxidative stress rapidly increases the level of regulatory subunit of PI 3-kinase and the PI 3-kinase activity in the immune complexes of occludin. These results demonstrate that PI 3-kinase does interact with tight junction complex, and it can be increased by physiologic or pathophysiologic conditions such as oxidative stress. This observation was further confirmed by confocal immunofluorescence localization of p85 at the junctions. Oxidative stress increased the localization of p85 at the intercellular junctions, with a concomitant decrease in p85 stain at the intracellular compartments. Double staining for p85 and occludin indicates that p85 is co-localized with occludin. At 5 min of XO+X treatment the PI 3-kinase activity associated with occludin was found to be higher than control in both ACS and TS fraction, suggesting an activation of PI 3-kinase at the early time period. Therefore, it possible that the initial effect of oxidative stress is activation of PI 3-kinase bound to occludin, which was immediately followed by a translocation of PI 3-kinase into occludin.
The binding of PI 3-kinase to occludin is not necessarily mean that it is associated with the tight junction. However, our previous study (20) showed that oxidative stress-induced increase in paracellular permeability and its reduction by genistein correlated well with the changes in the levels of actin-bound occludin. Changes in occludin present in MCS fraction or TS fraction did not correlate with the permeability changes, which indicated that actin-bound occludin is the pool of occludin that is most relevant to the tight junctions. Therefore, it is likely that PI 3-kinase activity present in occludin immunoprecipitates prepared from the ACS fraction is associated with the tight junction.

The rapid increase in the activity of PI 3-kinase in the tight junctions strongly indicates that this pool of PI 3-kinase plays a crucial role in the oxidative stress-induced disruption of tight junctions. However, it is not clear if the co-immunoprecipitation of p85 with occludin is a direct interaction. As immune complexes of occludin prepared under native conditions are expected to co-precipitate many of the tight junction proteins, it is possible that PI 3-kinase interacts indirectly with the occludin through other proteins. Therefore, we investigated the direct interaction of occludin with p85 by GST pull down assays using GST-C-Occludin and thrombin-cleaved GST-p85. Concentration-related binding of recombinant p85 with recombinant GST-C-occludin demonstrates that p85 directly interacts with the C-terminal tail of occludin. These studies suggest a possibility of direct interaction of p85 with occludin within the cell, and that this interaction is enhanced by oxidative stress. Such an enhancement of interaction between p85 and occludin may involve post-translational modifications, such as phosphorylation.

In summary, this study shows that oxidative stress increases the association of PI 3-kinase regulatory subunit and PI 3-kinase activity with the tight junction protein.
complex, and that PI 3-kinase activity is required for the oxidative stress-induced disruption of tight junctions.
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FIGURE LEGENDS

Fig. 1: Effect of PI 3-kinase inhibitors on oxidative stress-induced increase in paracellular permeability.  A: Caco-2 cell monolayers were incubated without (O) with XO+X (○ & □) in the absence (○) or presence of 25 μM LY294002 (□) for varying times.  TER was recorded at varying times.  Values are mean ± sem (n = 8).  B: Unidirectional flux of FITC-inulin measured at 2 hours after treatment with XO+X in the absence or presence of inhibitor.  Values are mean ± sem (n = 5), and asterisks indicate the values that are significantly (p<0.05) different from values for XO+X.  C & D: After 2 hour treatment with XO+X in the absence or presence of varying concentrations of LY294002.  TER (C) and inulin flux (D) were measured.  Values are mean ± sem (n = 3), and asterisks indicate the values that are significantly (p<0.05) different from values for XO+X.

Fig. 2: Effect of LY294002 on oxidative stress-induced redistribution of occludin and ZO-1.  Caco-2 cell monolayers were incubated without (Control) or with XO+X in the absence (XO+X) or presence of 25 μM LY294002 (XO+X + LY294002) for 2 hours.  Cell monolayers were fixed and stained for occludin and ZO-1 by immunofluorescence method.  Confocal fluorescence images were collected.

Fig. 3: Effect of PI 3-kinase inhibitors on oxidative stress-induced tyrosine phosphorylation and dissociation from the actin cytoskeleton of tight junction proteins.  A: Caco-2 cell monolayers were incubated with XO+X for varying times or for 60 min in the presence of 25 μM LY294002.  The actin cytoskeleton (ACS), the membrane
cytoskeleton (MCS) and Triton-soluble fraction (TS) were prepared and p-Tyr was immunoprecipitated. Immune complexes of p-Tyr were immunoblotted for occludin and ZO-1. B: Caco-2 cell monolayers were incubated with XO+X for varying times or for 60 min in the presence of 25 μM LY294002. The actin cytoskeleton (ACS), the membrane cytoskeleton (MCS) and Triton-soluble fraction (TS) were prepared and immunoblotted for occludin and ZO-1.

Fig. 4: Oxidative stress-induced redistribution and activation of PI 3-kinase. A: Caco-2 cell monolayers were incubated with XO+X for varying times. The actin cytoskeleton (ACS), the membrane cytoskeleton (MCS) and Triton-soluble fraction (TS) were prepared and immunoblotted for regulatory subunit of PI 3-kinase. B: Densitometric analysis of p85 bands for ACS calculated as percent of total densities (ACS+MCS+TS) shown in panel A. Average values from two independent experiments are presented. C: PI 3-kinase activity was measured as described in Methods section in the actin cytoskeleton (ACS) and Triton-soluble (TS) samples prepared from cell monolayers treated with XO+X for varying times. D: Densitometric analysis of phosphatidylinositol 3, 5-bisphosphate (PIP2) bands in PI 3-kinase assay for ACS calculated as percent of total (ACS+TS) as shown in panel C. Values are average of values from two independent experiments.

Fig. 5: The effect of oxidative stress on co-immunoprecipitation of PI 3-kinase with occludin. A: Caco-2 cell monolayers were incubated with XO+X for varying times. Under native extraction conditions occludin was immunoprecipitated from the actin
cytoskeleton and immunoblotted for p85 and occludin. B: Immunoblots in panel A were subjected to densitometric analysis and values are presented as the density of p85 to density of occludin ratio from corresponding experiments. Values are mean ± sem (n = 3). C: Caco-2 cell monolayers were incubated with XO+X for varying times. Under native extraction conditions occludins from actin cytoskeleton (ACS) and Triton-soluble (TS) fractions were immunoprecipitated. PI 3-kinase activity was measured in the immune complexes using \(^{32}\text{P}-\text{ATP}\) as described in Methods section. Autoradiography shows the amount of PIP2 generated in the assay system. D: Autoradiograms of PI 3-kinase assay in occludin immune complexes from ACS (■) and TS (●) as shown in panel C were subjected to densitometric analysis, and the values are presented as ratio of density of PIP2 bands (panel C) to density of occludin bands (blots not shown) from corresponding experiments. Values are mean ± sem (n = 3). E: Caco-2 cell monolayers were treated with XO+X for 60 min in the absence or presence of LY294002. PI 3-kinase activity was measured in occludin immune complexes prepared from the actin cytoskeleton (ACS) and triton-soluble (TS) fractions. F: Autoradiograms shown in E were subjected to densitometric analysis, and the values are presented as ratio of density for PIP2 bands and density for occludin (blots not shown) from corresponding experiments. Values are mean ± sem (n = 3).

Fig. 6: The effect of oxidative stress on translocation of p85 to the intercellular junction. Caco-2 cell monolayers were incubated with XO+X for varying times. Cell monolayers were fixed and stained for occludin and p85 by immunofluorescence method. Confocal fluorescence images were collected. Panel B shows the images from control monolayers incubated without XO+X. Panel C shows the z-sections of these images.
Fig. 7: Pair wise direct binding of p85 to occludin. Thrombin-cleaved recombinant p85 (0.03 μM) was incubated with GST-C-Occludin (0.1-1.0 μM) or GST (0.2-1.0 μM) in the presence of GSH-agarose. Proteins bound to agarose beads were then immunoblotted for p85.

Fig. 8: Role of PI 3-kinase in oxidative stress on disruption of tight junction in MDCK cell monolayers. A: MDCK cell monolayers were incubated without (□, △) with XO+X (▲, ■) in the absence (△, ▲) or presence of 25 μM LY294002 (□, ■) for varying times. TER was recorded at varying times. Values are mean ± sem (n = 4). B: Unidirectional flux of FITC-inulin measured at 3 hours after treatment with XO+X in the absence or presence of LY294002. Values are mean ± sem (n = 4), and asterisks indicate the values that are significantly (p<0.05) different from values for XO+X. C: MDCK cell monolayers were incubated without (Control) or with XO+X in the absence (XO+X) or presence of LY294002 (XO+X + LY294002) for 3 hours. Cell monolayers were fixed and stained for occludin by immunofluorescence method. Confocal fluorescence images were collected.
Role of phosphatidylinositol in oxidative stress-induced disruption of tight junction

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