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Protein Phosphatase 2A Plays a Role in Hydrogen Peroxide-Induced Disruption of Tight Junctions in Caco-2 Cell Monolayers

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Keywords

occludin; epithelium; barrier function; cell-cell adhesion; phospho-threonine; c-Src

INTRODUCTION

An important function of the epithelial tight junction is to form a barrier to the diffusion of pathogens, toxins and allergens from the external environment into the tissues. The disruption of tight junction and increased permeability play an important role in the pathogenesis of numerous gastrointestinal, lung and kidney diseases [1–3]. Inflammatory mediators such as TNFα [4], IFNγ [5] and reactive oxygen species [6] are known to disrupt the tight junctions and barrier function in various epithelial monolayers. Previous studies demonstrated that hydrogen peroxide disrupts tight junctions in the intestinal epithelium without affecting the cell viability [7]. Therefore, understanding the mechanisms involved in tight junction disruption by oxidative stress is important in delineating the mechanisms involved in the pathogenesis of various inflammatory diseases.

The tight junction is organized by specific interactions between a wide-spectrum of proteins. Four types of transmembrane proteins, occludin, claudins [8], tricellulin [9] and junctional adhesion molecule [10] 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. A significant body of evidence indicates that the activities of various intracellular signaling molecules regulate the integrity of tight junctions. The signaling pathways involving protein kinases, G-proteins, Rho/Rac GTPases regulate the tight junction permeability in different epithelial monolayers [7, 11–20]. A number of previous studies showed that oxidative stress disrupts epithelial tight junctions and increases the paracellular permeability in Caco-2 cell monolayer [6, 7, 12, 16, 17, 21, 22]. Oxidative stress-induced disruption of tight junctions in Caco-2, T84 and MDCK cell monolayers involves tyrosine kinase activity [6, 7, 12, 16, 17, 21, 22]. Recent studies demonstrated that c-Src [12] and phosphatidylinositol 3-kinase [22] are involved in the oxidative stress-induced disruption of tight junction in Caco-2 and MDCK cell monolayers. Oxidative stress-induced disruption of tight junction is associated

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with Tyr-phosphorylation of occludin [7, 16], and that Tyr-phosphorylation of occludin alters its interaction with ZO-1, ZO-2 and ZO-3 [23].

Occludin has been shown to be highly phosphorylated on Ser and Thr residues [24–26], and it undergoes dephosphorylation during the disruption of tight junctions by calcium depletion, phorbol esters and bacterial infection [27, 28]. Phosphorylation of this protein may be mediated by the atypical protein kinase C (PKC), such as PKCζ and PKCλ, or PKCη [29] as these PKC isoforms were shown to be associated with the tight junctions [19]. Recent studies indicated that PP2A, a Ser/Thr-phosphatase, interacts with the tight junction protein complex and suggested that it may negatively regulate the integrity of tight junctions in MDCK and Caco-2 cell monolayers [30, 31].

In the present study, we examined the role of PP2A in hydrogen peroxide-induced disruption of tight junction in Caco-2 cell monolayers. Results show that: 1) hydrogen peroxide induces translocation of PP2A to the detergent-insoluble fraction and dephosphorylates occludin on Thr residues, 2) okadaic acid and fostriecin (selective PP2A inhibitors) attenuate hydrogen peroxide-induced disruption of tight junction and increase in paracellular permeability, 3) reduced expression of PP2A-Cα by antisense oligonucleotides or siRNA significantly ameliorates hydrogen peroxide-induced disruption of tight junction and increase in paracellular permeability, 4) hydrogen peroxide enhances the association of PP2A with tight junction-protein complex by a Src kinase-dependent mechanism, and 5) PP2A directly interacts with the C-terminal domain of occludin, which was enhanced by Tyr-phosphorylation of occludin by c-Src.

EXPERIMENTAL

Chemicals

Cell culture reagents and supplies were purchased from GIBCO-BRL (Grand Island, NY). FITC-inulin, vanadate, SDS, hydrogen peroxide, protease inhibitors, streptavidin agarose, protein-A Sepharose and protein-G Sepharose were purchased from Sigma Chemical Company (St Louis, MO). Okadaic acid, fostriecin and PP2 were purchased from Calbiochem (San Diego, CA). All other chemicals were of analytical grade purchased either from Sigma Chemical Company or Fisher Scientific (Tustin, CA). Purified PP2A was purchased from Millipore (Bellerica, MA).

Antibodies

Mouse monoclonal anti-PP2A-Cα, anti-GST, anti-PP1, recombinant HRP-conjugated anti-p-Tyr, anti-mouse IgG and HRP-conjugated anti-rabbit IgG antibodies were purchased from BD Transduction Laboratories (San Jose, CA). Mouse monoclonal anti-occludin and rabbit polyclonal anti-ZO-1, anti-occludin, anti-p-Thr and anti-p-Ser antibodies, and HRP-conjugated anti-occludin antibodies were purchased from Zymed Laboratories (San Francisco, CA). Cy3-conjugated anti-rabbit IgG and AlexaFluor 488-conjugated anti-mouse IgG antibodies were purchased from Molecular Probes (Eugene, OR).

Cell culture

Caco-2 cells purchased from American Type Cell Collection (Rockville, MD) were grown under standard cell culture conditions as described before [12]. 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.

Antisense oligonucleotides and siRNA

Antisense oligos targeting human PP2A-Cα nucleotide sequence and corresponding missense oligonucleotide were designed and custom synthesized as described previously [31]. Out of four different antisense oligos two oligos (AS-PP22 and AS-PP23) were found to be very effective in reducing PP2A-Cα expression. FITC-conjugated antisense oligo was synthesized to visualize the transfection efficiency in Caco-2 cells. PP2A-specific siRNA smart pool and control RNA with scrambled sequence were purchased from Dharmacon (Lafayette, CO). Individual siRNAs in the smart pool were tested separately, and three out of four siRNAs (si-PP21, si-PP22 & si-PP23) were found to be very effective in reducing PP2A-Cα level.

Treatment with PP2A inhibitors

Caco-2 cells were treated with protein phosphatase inhibitors as previously described [32]. Cell monolayers were incubated with fostriecin (25–100 nM), okadaic acid (1–6 nM) and calyculin A (0.5 nM) for 24 hours prior to the experiment. The cells were then washed with DMEM and the inhibitors were added again during hydrogen peroxide treatment to maintain the inhibitory effect.

Transfection of antisense oligos and siRNA

Caco-2 cells (125,000 cells/well) were seeded in 6 well plates. The cells were then allowed to grow and attach for 24 hours. After the incubation, the cells were treated with serum free, antibiotic free DMEM and the incubation continued for an additional 24 hours. The cells were then transfected, using 1 ml antibiotic and serum free DMEM with or without 80 picomoles of the antisense oligonucleotides or siRNA and 3.15 μl of Oligofectamine® reagent in each well and incubated for 6 hours at 37˚C. Serum was then added to the medium to make a final concentration of 10% serum, and incubated at 37˚C. After 24 hours, the cell monolayers were trypsinized and seeded on to Transwell inserts, and the TER was monitored every day. Hydrogen peroxide treatment was performed on day 4. For controls, cells were transfected with missense oligos or control RNA with a scrambled nucleotide sequence at similar dose and transfection conditions.

Hydrogen peroxide treatment

Oxidative stress was induced as previously described [16]. 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 20 μM hydrogen peroxide. Control cell monolayers were incubated in PBS without hydrogen peroxide.

Measurement of transepithelial electrical resistance (TER)

TER was measured as described previously [16] using a Millicell-ERS Electrical Resistance System (Millipore, Bedford, MA). TER was calculated as Ohms.cm^2 by multiplying it with the surface area of the monolayer. The resistance of the polycarbonate membrane in Transwells (approximately 30 Ohms.cm^2) 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 hydrogen peroxide 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 cm^2 surface area.

Immunofluorescence Microscopy

After treatment with hydrogen peroxide in the absence or presence of inhibitors 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 (mouse monoclonal anti-occludin and rabbit polyclonal anti-ZO-1), followed by incubation for one hour with secondary antibodies (AlexaFluor 488-conjugated anti-mouse IgG and Cy3-conjugated anti-rabbit IgG). The fluorescence was visualized using a Zeiss LSM 5 Laser Scanning Confocal Microscope and the images from Z-series sections (1 μm) collected by using Zeiss LSM 5 Pascal, the confocal microscopy software (Release 3.2). Images were stacked using the software, Image J (NIH), and processed by Adobe Photoshop (Adobe Systems Inc., San Jose, CA).

Preparation of detergent-insoluble fraction

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 × 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 and sonicated at 0°C for 10 sec. Protein contents in different fractions were measured by BCA method (Pierce Biotechnology Inc., Rockford, IL). Triton-insoluble and Triton-soluble fractions were mixed with equal volume of Laemmli's sample buffer (2× concentrated) and heated at 100°C for 5 min.

Immunoprecipitation

For co-immunoprecipitation studies, Caco-2 cell monolayers (24 mm) were washed with ice-cold 20 mM Tris (pH 7.4) and Triton-insoluble cytoskeleton and Triton-soluble fraction were prepared. The suspension of Triton-insoluble fraction was sonicated at 0°C for 10 seconds in lysis buffer-N (20 mM Tris, pH 7.4, containing 0.2% NP40, 0.1% sodium deoxycholate and a cocktail of protease inhibitors) to fragment the F-actin filaments and

release the bound protein complexes. Homogenate was centrifuged at 15,600 × g for 30 min to pellet the F-actin fragments. The supernatant from the homogenate of Triton-insoluble fraction and the 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 μl of assay buffer to measure PP2A activity, or heated in Laemmli's sample buffer for immunoblot analysis for PP2A-Cα.

For Ser/Thr phosphorylation studies, total proteins from the cells 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 and centrifuged to remove undissolved particles. Supernatants (1 mg protein/ml) were incubated for 16 hours at 4°C with 2 μg of rabbit polyclonal anti-p-Thr or anti-p-Ser antibodies. Immunocomplexes were precipitated by incubation for one hour with protein-A Sepharose at 4°C. Immune complexes were then immunoblotted for occludin.

Immunoblot analysis

Proteins were separated by SDS-polyacrylamide gel (4–12 % gradient) electrophoresis and transferred to PVDF membranes. Membranes were blotted for occludin, ZO-1, p-Tyr, PP2A-Cα and PP1 by using specific antibodies in combination with HRP-conjugated anti-mouse IgG or HRP-conjugated anti-rabbit IgG antibodies. The blots were developed by using the ECL chemiluminescence method (Amersham, Arlington Heights, IL).

PP2A assay

Anti-occludin immunocomplexes were diluted in PPase buffer (50 mM HEPES, pH 7.2, 60 mM NaCl, 60 mM KCl, and protease inhibitors) to a final volume of 20 μl and incubated with 5 μl phosphopeptide substrate, KR**pT**IRR (5 μg). After incubation at 30°C for 10 min, free phosphate was assayed by adding 100 μl Malachite green reagent to each sample in a 96-well microtiter plate. After 10 minutes of incubation at 30°C, absorbance was measured at 650 nm wavelength in a microplate reader (SpectraMax 190, Molecular Devices, Sunnyvale, CA). Zero minute incubation was used for the control assay. Assay was also performed in the presence of 100 nM fostriecin. To determine PP2A-specific activity, the phosphatase activity measured in the presence of 100 nM fostriecin was subtracted from the corresponding total activity. The unit (U) of PP2A activity represents picomole of free phosphate generated in 1 hour under assay conditions.

Preparation of GST-Occludin-C

C-terminal tail of chicken occludin as a GST fusion protein (GST-Occludin-C), was prepared in E. coli DH5α cells and purified using GSH-Agarose as described before [23]. cDNA for C-terminal tail of human occludin (C-terminal 150 amino acids) in pGEX vector was generated by PCR cloning using full length human occludin cDNA (a gift from Dr. Van Italie, University of North Carolina, Chapel Hill, NC).

Tyr-phosphorylation of GST-Occludin-C

Recombinant GST-Occludin (5 μg) was incubated with 500 ng of active c-Src in 20 μl kinase buffer (50 mM Hepes pH 7.4, 1 mM EDTA, 0.2% β-mercaptoethanol, 3 mM $MgCl_2$) containing 100 μM ATP at 30°C for 3 hours on a shaking incubator. Control reactions were done in the absence of ATP.

Pair wise binding assay—This particular assay detects binding between two individually purified proteins. Binding assays were performed using GST-Occludin-C and purified PP2A (dimer of A and C subunits), and glutathione-Sepharose was used to 'pull down' the bound complex. Complexes were then immunoblotted for PP2A-Cα. This assay determines direct interaction between two proteins.

RESULTS

Hydrogen peroxide induces dephosphorylation of occludin on Thr residues in Caco-2 cell monolayer.

Occludin is highly phosphorylated on Ser and Thr residues in normal epithelium [24–26], which is dephosphorylated during tight junction disruption by various factors [27, 28]. Therefore, the effect of hydrogen peroxide on Ser/Thr-phosphorylation of occludin was determined. Immunoprecipitation of p-Thr and p-Ser followed by immunoblot analysis for occludin demonstrated that occludin is highly phosphorylated on both Ser and Thr residues in Caco-2 cell monolayers. Hydrogen peroxide rapidly reduced the level of Thr-phosphorylated occludin (Fig. 1A); Thr-phosphorylation of occludin in control cells remained unaffected up to 2 hours of incubation. Phosphorylation of occludin on Ser residues was also reduced, but transiently, by hydrogen peroxide treatment (Fig. 1B).

Hydrogen peroxide induces translocation of PP2A to the detergent-insoluble fraction

Reversible phosphorylation of proteins on Ser/Thr residues is mediated by protein kinases and protein phosphatases. Inhibition of protein kinases and activation or subcellular translocation of protein phosphatases may be involved in the hydrogen peroxide-induced dephosphorylation of occludin on Thr residues. Okadaic acid (the selective inhibitor of PP2A)-sensitive protein phosphatase activity was detected in both Triton-insoluble and soluble fractions. Treatment with hydrogen peroxide significantly increased PP2A activity in Triton-insoluble fractions, while activity in Triton-soluble fraction was significantly reduced by hydrogen peroxide (Fig. 1C). This effect of hydrogen peroxide was concentration-dependent (Fig. 1D).

Inhibition of PP2A activity attenuates hydrogen peroxide-induced disruption of tight junction and increase in paracellular permeability

Previous studies have indicated that PP2A is associated with the tight junction protein complex of MDCK and Caco-2 cell monolayers [30, 31] and that PP2A-selective inhibitors, okadaic acid and fostreicin accelerate the assembly of tight junctions [31]. In the present study, we evaluated the effect of okadaic acid and fostriecin on hydrogen peroxide-induced disruption of tight junction and increase in paracellular permeability. Incubation of Caco-2 cells with varying concentrations of okadaic acid or fostriecin significantly reduced the

activity of PP2A in a dose-dependent manner without affecting the levels of PP2A-Cα protein [31]. Okadaic acid and forstriecin slightly, but significantly increased the baseline TER (Fig. 2A). Pretreatment with okadaic acid or fostriecin significantly attenuated hydrogen peroxide-induced decrease in TER (Fig. 2A) and increase in inulin permeability (Fig. 2B). The effects of okadaic acid and fostriecin on hydrogen peroxide-induced decrease in TER (Fig. 2C) and inulin flux (Fig. 2D) were concentration-dependent.

Confocal immunofluorescence microscopy demonstrated that occludin and ZO-1 were co-localized at the intercellular junctions in control cell monolayers (Fig. 3). Hydrogen peroxide induced redistribution of occludin and ZO-1 from the intercellular junctions into the intracellular compartments (Fig. 3). Pretreatment of cell monolayers with fostriecin prevented hydrogen peroxide-induced redistribution of occludin and ZO-1.

Reduced expression of PP2A-Cα ameliorates hydrogen peroxide-induced disruption of tight junction and increase in paracellular permeability

To reduce the levels of PP2A, Caco-2 cells were transfected with antisense oligonucleotides (AS-PP22 and AS-PP23) or siRNA (si-PP21, si-PP22 and si-PP23) designed against the nucleotide sequence of human PP2A-Cα (the catalytic subunit). Confocal microscopy of fluorescein-conjugated AS-PP23 demonstrated that considerable levels of antisense oligo were present in the intracellular compartments 2 hour after incubation with the oligos (data not shown). Transfection of two different antisense oligos (AS-PP22 and AS-PP23) reduced the expression of PP2A-Cα, without altering the levels of PP1 (Fig. 4A). Transfection with antisense oligos slightly, but significantly, increased the baseline TER (data not shown). Transfection with either AS-PP22 or AS-PP23 significantly attenuated the hydrogen peroxide-induced decrease in TER as compared to transfection with missense oligos (Fig. 4B). There were no significant differences in the TER of cell monolayers transfected with antisense and missense oligos during incubation in the absence of hydrogen peroxide (Fig. 4C).

Hydrogen peroxide treatment increased inulin permeability in non-transfected and missense oligo-transfected cells, while transfection with AS-PP22 or AS-PP23 significantly reduced the hydrogen peroxide-induced increase in inulin permeability (Fig. 4D). Transfection of cells with AS-PP22 and AS-PP23 together was much more effective in reducing the hydrogen peroxide-induced changes in TER (Fig. 4B) and inulin permeability (Fig. 4D).

Transfection of cells with siRNA to PP2A-Cα (si-PP21, si-PP22 and si-PP23) also effectively reduced the expression of PP2A-Cα without affecting the levels of PP1α (Fig. 4E). Transfection with siRNA induced slight increase in basal TER and decrease in inulin permeability (data not shown). All three siRNAs significantly attenuated hydrogen peroxide-induced increase in inulin permeability (Fig. 4F). Hydrogen peroxide reduced the Thr-phosphorylation of occludin in missense oligo-transfected cell monolayers, and this effect of hydrogen peroxide on occludin dephosphorylation was attenuated in si-PP23-transfected cell monolayers (Fig. 4G). Hydrogen peroxide induced redistribution of occludin and ZO-1 from the intracellular junctions into the intracellular compartments in control RNA-transfected cell monolayers (Fig. 5A). However, hydrogen peroxide failed to induce redistribution of occludin and ZO-1 in si-PP23-transfected cell monolayers. These observations were

confirmed by quantitating the fluorescence intensity for occludin (Fig. 5B) and ZO-1 (Fig. 5C) at the junctional and intracellular compartments. Transfection of si-PP23 by itself appeared to enhance junctional distribution of occludin and ZO-1, suggesting that basal PP2A activity may render negative impact on tight junction integrity as well.

Hydrogen peroxide-enhances the association of PP2A with tight junction protein complex by a Src kinase-dependent mechanism

A previous study demonstrated that PP2A associates with tight junction protein complex in Caco-2 cell monolayers [31]. In the present study we evaluated the effect of hydrogen peroxide on the association of PP2A with tight junction protein complex this was evaluated by measuring PP2A protein level in anti-occludin immunocomplexes prepared from the Triton-insoluble and Triton-soluble fractions. Immunoblot analysis showed that a considerable amount of PP2A-Cα protein was associated with the anti-occludin immunocomplexes prepared from control cell monolayers (Fig. 6A). Hydrogen peroxide treatment significantly increased the level of occludin-associated PP2A-Cα in the detergent-insoluble fractions (Fig. A and B). The PP2A activity associated with anti-occludin immunocomplexes was also rapidly increased by hydrogen peroxide treatment (Fig. 6C). The basal and hydrogen peroxide-induced levels of occludin-associated PP2A activity were significantly reduced by the treatment of cells with okadaic acid and fostriecin (Fig. 6D). Confocal immunofluorescence microscopy showed that treatment of cells with hydrogen peroxide for 60 min resulted in increased localization of PP2A-Cα at the tight junctions and its increased co-localization with occludin (Fig. 6E).

A previous study demonstrated that hydrogen peroxide induces a rapid activation of c-Src, and that c-Src activity is required for hydrogen peroxide-induced disruption of tight junction and increase in paracellular permeability [12]. Previous studies also showed that hydrogen peroxide-induced disruption of tight junction is associated with Tyr-phosphorylation of occludin [16]. In the present study, we evaluated the role of Src kinase in hydrogen peroxide-induced increase in PP2A activity in anti-occludin immunocomplexes. Pretreatment of cells with PP2, a selective inhibitor of Src kinase, prevented hydrogen peroxide-induced increase in occludin-associated PP2A activity (Fig. 6F & 6G). Src inhibitor by itself produced no significant effect on PP2A activity.

PP2A directly interacts with the C-terminal domain of occludin by a phosphorylation-dependent mechanism

Above studies indicate that hydrogen peroxide enhances the PP2A association with occludin by a Src kinase-dependent mechanism, and our recent study demonstrated that PP2A directly interacts with the occludin C-terminal domain [31]. Another previous study demonstrated that c-Src activation and occludin tyrosine phosphorylation play a crucial role in hydrogen peroxide-induced disruption of barrier function in Caco-2 cell monolayers [12]. Therefore, we evaluated the effect of c-Src-induced Tyr-phosphorylation of occludin C-terminal domain on its direct interaction with PP2A. Incubation of GST-Occludin-C with c-Src in the presence of ATP induced Tyr-phosphorylation of occludin (Fig. 7A). Binding of GST-Occludin-C to PP2A was determined by incubation with purified PP2A followed by GST pull down assay. PP2A binds to Tyr-phosphorylated GST-Occludin-C in a dose-

dependent manner (Fig. 7B), while binding of PP2A to non-phosphorylated GST-Occludin-C and GST was undetectable under the present experimental conditions. Src did induce Tyr-phosphorylation of GST to some extent (Fig. 7A). However, both Tyr-phosphorylated and non-phosphorylated GST failed to bind PP2A (Fig. 7B).

DISCUSSION

Association of hyper phosphorylated occludin with the detergent-insoluble fractions of cells [24–26] and its dephosphorylation during the disruption of tight junction by various factors [27, 28] suggest that phosphorylation of occludin on Ser/Thr residues may play an important role in the regulation of the epithelial tight junctions. In the present study, we provide evidence that occludin undergoes dephosphorylation on Thr residues, but not on Ser residues, during the hydrogen peroxide-induced disruption of tight junction in Caco-2 cell monolayers, and that translocation of PP2A to tight junctions plays a crucial role in hydrogen peroxide-induced disruption of tight junction and increase in paracellular permeability. The present study also demonstrates that hydrogen peroxide enhances the association of PP2A with the tight junction by a Src kinase-dependent mechanism.

Immunoprecipitation of p-Thr followed by immunoblot analysis for occludin showed that hydrogen peroxide-induced disruption of tight junction and increase in paracellular permeability is associated with the dephosphorylation of occludin on Thr residues. On the other hand, phosphorylation of occludin on Ser residues was slightly and transiently increased by hydrogen peroxide. Therefore, the regulation of Thr-phosphorylation of occludin may play an important role in the hydrogen peroxide-induced disruption of tight junction. High levels of phosphorylation of occludin on Ser and Thr residues were shown in MDCK I [24], LLC-PK1 [27], MDCK II [25], T84 [28] and Caco-2 [29, 31] cells. This hyper phosphorylated occludin is localized predominantly in the detergent-insoluble fractions of cells. In MDCK I cells, occludin is phosphorylated predominantly on Ser residues than in Thr residues; occludin isolated from detergent-insoluble fraction was found to be phosphorylated exclusively on Ser residues [24]. Similarly, a later study demonstrated that 62–65 kDa occludin band was phosphorylated on both Ser and Thr residues, while the 71 kDa band was phosphorylated exclusively on Ser residues [25]. Overall phosphorylation of 62–65 kDa and 71 kDa occludins were reduced by prolonged exposure to low calcium medium or treatment with phorbol esters [27]; disappearance of 71 kDa occludin indicated that dephosphorylation of p-Ser was more pronounced in this study. A more recent study demonstrated that phorbol ester-induced disruption of tight junction was associated with rapid decrease in Thr-phosphorylation of occludin in LLC-PK1 cells [27]. The specific function of Thr or Ser phosphorylation of occludin is not known. It may play a role in regulation of the interactions of occludin with other proteins of the tight junction complex. Our previous study has shown that tight junction disruption mediated by calcium depletion did not alter Ser-phosphorylation of occludin [31], while the present study shows a transient reduction in Ser-phosphorylation of occludin during tight junction disruption by hydrogen peroxide. Such a difference in occludin Ser-phosphorylation may be due to different signaling pathways involved in tight junction disruption under different conditions.

Both the inhibition of PP2A activity by selective PP2A-inhibitors (okadaic acid and fostriecin) and reduced expression of PP2A-Cα by antisense oligonucleotides or siRNA attenuated hydrogen peroxide-induced increase in paracellular permeability and disruption of tight junctions. Okadaic acid and fostriecin are well-established inhibitors of PP2A [32]. The present study demonstrates that incubation with okadaic acid or fostriecin reduces PP2A activity in Caco-2 cells and attenuates hydrogen peroxide-induced disruption of tight junction and increase in paracellular permeability. Antisense oligonucleotides and siRNA selectively reduced cellular levels of PP2A-Cα, without affecting the level of PP1. Therefore, PP2A activity appears to be required for hydrogen peroxide-induced disruption of tight junctions. This observation is complementary to our previous observation that PP2A-Cα negatively regulates the tight junction integrity in Caco-2 cell monolayers [31]. Therefore, the present study suggested that PP2A plays a role in hydrogen peroxide-induced dephosphorylation of occludin on Thr residues. Interestingly, our previous study also showed that PP2A preferentially dephosphorylated p-Thr in occludin compared to p-Ser.

Co-immunoprecipitation of PP2A-Cα with occludin in Caco-2 cells indicated that PP2A interacts with the tight junction protein complex in Caco-2 cells, which is in support with previous reports [30, 31]. The present study demonstrated that the association of both PP2A-Cα protein and its activity with the anti-occludin immuno-complexes was rapidly increased by hydrogen peroxide treatment, which was confirmed by confocal immunofluorescence microscopy. Pretreatment with okadaic acid or fostriecin prevented hydrogen peroxide-induced increase in PP2A activity in occludin immunocomplexes. These results suggest that hydrogen peroxide induces translocation of PP2A to the tight junction, suggesting that PP2A may play an important role in hydrogen peroxide-induced dephosphorylation of occludin on Thr residues. The mechanism of PP2A translocation is not clear and requires further studies. However, it is likely that phosphorylation of either PP2A subunits or tight junction proteins is involved in this regulation of PP2A translocation.

Our previous study demonstrated that oxidative stress rapidly activates the non-receptor tyrosine kinase, c-Src [12] and induces tyrosine phosphorylation of occludin by a c-Src-dependent mechanism [23]. Expression of dominant negative c-Src prevented oxidative stress-induced disruption of tight junction, indicating that c-Src plays an important role in oxidative stress-induced barrier disruption [12]. Therefore, we evaluated the effect of Src kinase inhibitors on hydrogen peroxide-induced increase in the association of PP2A activity with the anti-occludin immnuocomplexes. Data presented in this study indicates that hydrogen peroxide does induce translocation of PP2A to the tight junction by an Src kinase-dependent mechanism. The function of PP2A at the tight junction is not clear, but prevention of hydrogen peroxide-induced Thr-dephosphorylation of occludin by knockdown of PP2A-Cα indicated that PP2A does play a role in Thr-dephosphorylation of occludin. It is possible that PP2A also plays a role in dephosphorylation of other tight junction-proteins on Ser and/or Thr residues. It is not clear whether PP2A dephosphorylates occludin directly or if it is mediated by the activation of other phosphatases or inhibition of protein kinases.

A previous study suggested that PP2A interacts with PKCζ and inhibits its activity leading to reduced phosphorylation of occludin and ZO-1 [30]. Our recent study using recombinant occludin demonstrated that PP2A does directly interact with occludin C-terminal domain

and dephosphorylates it *in vitro* on Thr residues [31]. Therefore, it is likely that PP2A directly binds to the C-terminal domain of occludin and dephosphorylates occludin in hydrogen peroxide-treated cells. As we used a dimer of A and C subunits of PP2A in the *in vitro* binding studies, it is not clear if PP2A binds through A or C subunit. However, it is clear that the B subunit is not required for PP2A interaction with occludin. The present study shows that *in vitro* incubation with c-Src induces Tyr-phosphorylation of GST-fused C-terminal domain of occludin. PP2A binding studies demonstrated that c-Src-mediated Tyr-phosphorylation of occludin C-terminal domain results in enhanced binding of PP2A. Although it is not clear if Src directly increases the Tyr-phosphorylation of occludin in the cell, c-Src was found to be required for oxidative stress-induced Tyr-phosphorylation of occludin [12]. Therefore, it is likely that increased Tyr-phosphorylation of occludin by hydrogen peroxide may have enhanced the interaction of PP2A with occludin. PP2A interaction with tight junction may result in dephosphorylation of occludin on Thr residues directly. Our previous study also indicated that occludin phosphorylation on Tyr residues attenuates its interaction with ZO-1 [23, 33]. Therefore, question arises whether binding of PP2A requires tyrosine phosphorylation-mediated dissociation of ZO-1. Although it is a likely scenario, it is hard to conclude on the basis of currently available information.

In summary, this study shows that hydrogen peroxide enhances translocation of PP2A by a Src kinase-dependent mechanism, leading to dephosphorylation of occludin on Thr residues and disruption of tight junction and increase in paracellular permeability.

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Abbreviations:

PBS	phosphate buffered saline
HRP	horse radish peroxidase
SDS	sodium dodecyl sulfate
PMSF	phenylmethyl sulfonyl fluoride
ZO-1, ZO-2 & ZO-3	zonula occludens 1, 2 & 3
TER	transepithelial electrical resistance
PP2A	protein phosphatase 2A
PP1	protein phosphatase 1
PKC	protein kinase C
Ser	serine
Thr	threonine
Tyr	tyrosine

GST	glutathione S-transferase
GST-Occludin-C	GST-conjugated C-terminal tail of occludin
p-Thr	phospho-threonine
p-Ser	phospho-serine
p-Tyr	phospho-tyrosine

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SYNOPSIS

Evidence indicates that PP2A interacts with epithelial tight junctions and negatively regulates the integrity of tight junction. In the present study, the role of PP2A in hydrogen peroxide-induced disruption of tight junction was examined in Caco-2 cell monolayers. Hydrogen peroxide-induced decrease in electrical resistance and increase in inulin permeability was associated with the dephosphorylation of occludin on Thr residues. The hydrogen peroxide-induced decrease in electrical resistance, increase in inulin permeability and redistribution of occludin and ZO-1 from the intercellular junctions were significantly attenuated by selective inhibitors of PP2A (okadaic acid and fostriecin) and by knockdown of PP2A-Cα (the catalytic subunit of PP2A). The PP2A-Cα protein and the PP2A activity were co-immunoprecipitated with occludin, and this co-immunoprecipitation was rapidly increased by hydrogen peroxide. Hydrogen peroxide-induced increase in co-immunoprecipitation of PP2A-Cα with occludin was prevented by PP2, a Src kinase inhibitor. GST-pull down assays using recombinant GST-Occludin-C (the C-terminal 150 amino acids) and the purified PP2A showed that PP2A binds to the C-terminal domain of occludin; Src-induced Tyr-phosphorylation of GST-Occludin-C enhanced this binding. This study shows that hydrogen peroxide increases the association of PP2A with the occludin by a Src kinase-dependent mechanism, and that PP2A activity is involved in hydrogen peroxide-induced disruption of tight junction in Caco-2 cell monolayers.

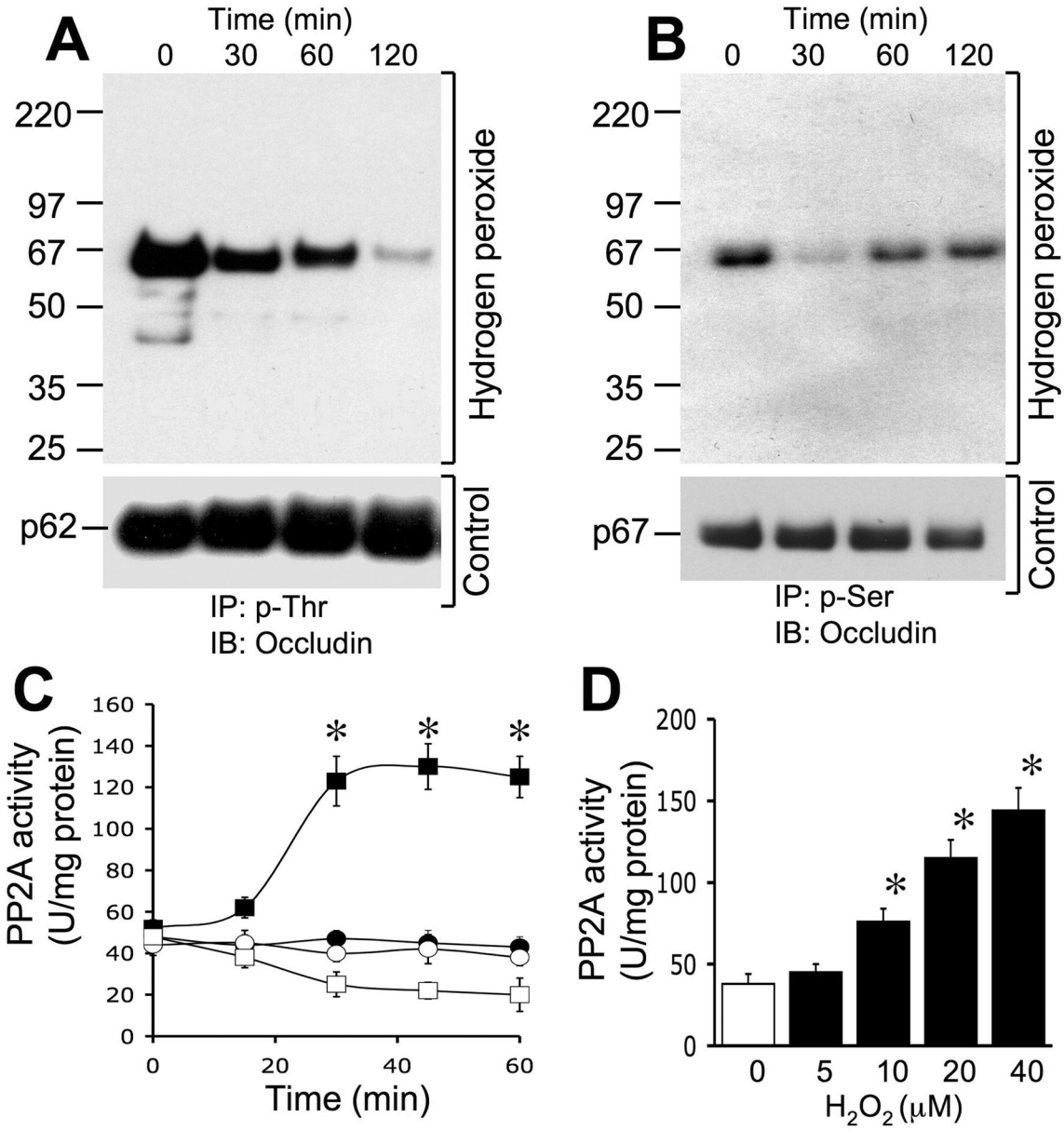

Fig. 1: hydrogen peroxide induces dephosphorylation of occludin on Thr residues and translocation of PP2A to the detergent-insoluble fractions.

A & B: Caco-2 cell monolayers were incubated with or without hydrogen peroxide (20 μM) for varying times. Proteins extracted under denatured conditions were subjected to immunoprecipitation (IP) of p-Thr (A) or p-Ser (B), the immunocomplexes were immunoblotted (IB) for occludin. The numbers on the left side of the blots represent the location and molecular weight of standard proteins; p62 represents the bands for occludin. **C:** Caco-2 cell monolayers were incubated with (squares) or without (circles) hydrogen peroxide (20 μM) for varying times. Triton-insoluble (solid symbols) and soluble (open symbols) fractions were prepared and PP2A activity was measured. Values are mean ± sem (n = 6). Asterisks indicate the values that are significantly ($P<0.05$) different from zero time values (C). **D:** PP2A activity in Triton-insoluble fractions was measured in cells treated with

varying doses of hydrogen peroxide for 60 min. Values are mean ± sem (n = 6). Asterisks indicate the values that are significantly ($P<0.05$) different from values for no hydrogen peroxide (D).

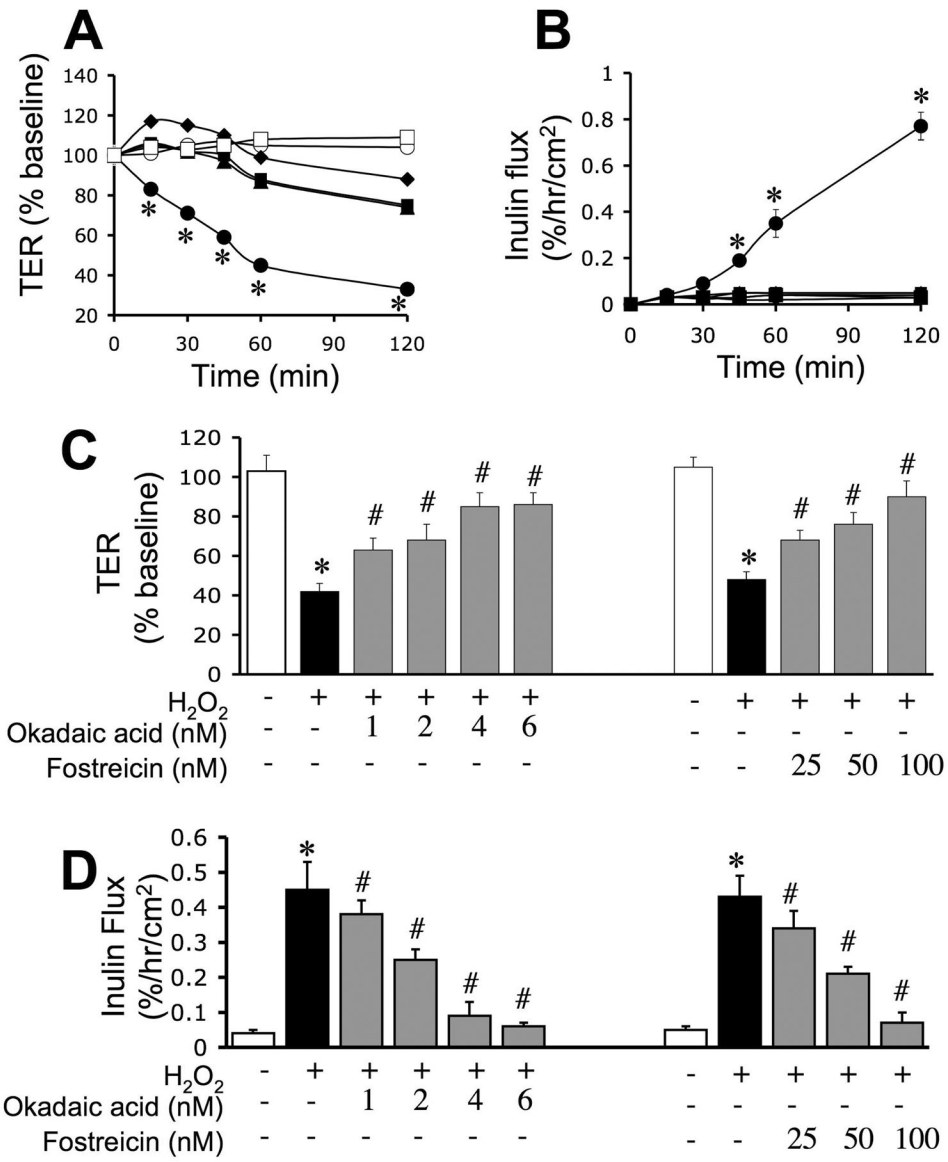

Fig. 2: Protein phosphatase activity is involved in hydrogen peroxide-induced increase in paracellular permeability.

A & B: Caco-2 cell monolayers were pretreated with 6 nM okadaic acid (■), 0.5 μM calyculin A (▲) or 50 nM fostriecin (◆) prior to incubation with hydrogen peroxide (20 μM) for varying times. Control cell monolayers were incubated with (□) or without (○) fostreicin in the absence of hydrogen peroxide. TER (**A**) and inulin flux (**B**) were measured at varying times. Values are mean ± sem (n = 6). Asterisks indicate the values that are significantly ($P<0.05$) different from corresponding values fro other groups. **C & D:** Effect of different doses of okadaic acid and fostriecin on TER (**C**) and inulin flux (**D**). Values are mean ± sem (n = 6). Asterisks indicate the values that are significantly ($P<0.05$) different from corresponding control values. Symbol # indicate the values that are significantly ($P<0.05$) different from value for corresponding hydrogen peroxide group.

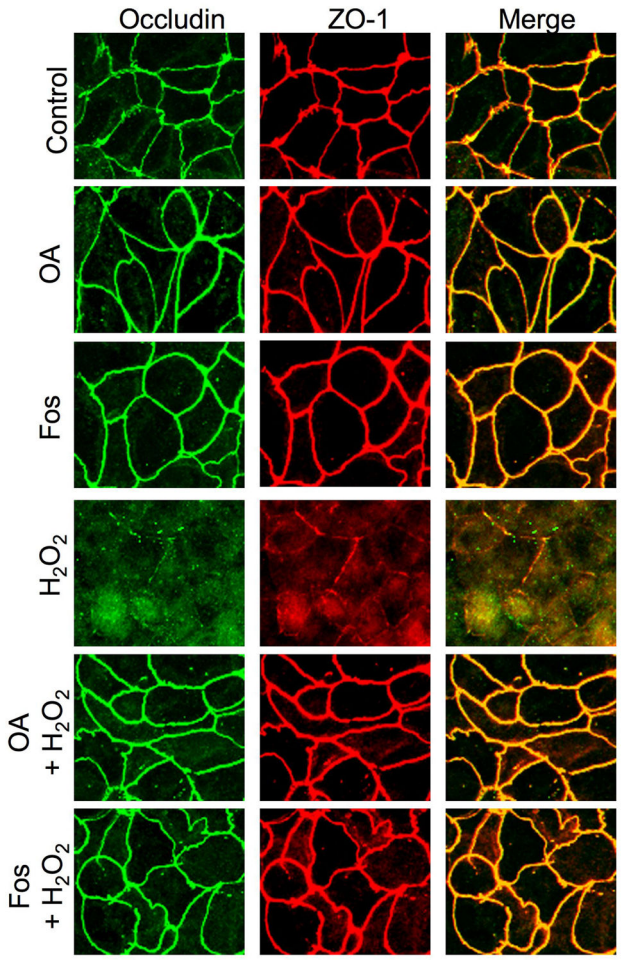

Fig. 3: Inhibition of PP2A activity attenuates the hydrogen peroxide-induced disruption of tight junctions.

Caco-2 cell monolayers were pretreated with 6 nM okadaic acid (OA) or 50 nM fostriecin (Fos) prior to incubation with hydrogen peroxide (20 μM) for 2 hours. Fixed cell monolayers were stained for occludin and ZO-1 by immunofluorescence method and images collected by confocal microscopy.

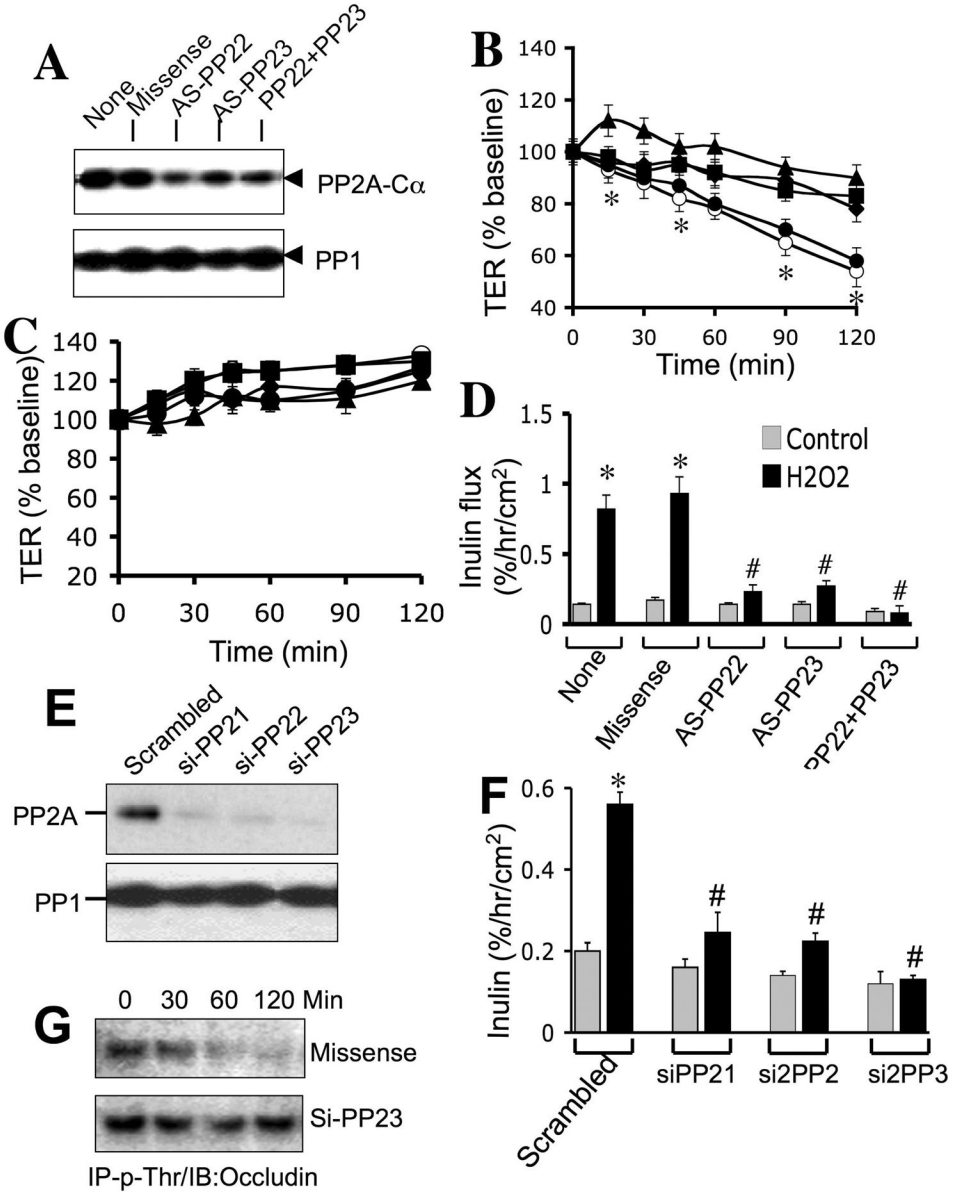

Fig. 4: Knockdown of PP2A-Cα attenuates hydrogen peroxide-induced disruption of barrier function.

Caco-2 cell monolayers were transfected with antisense oligos (AS-PP22, AS-PP23 or AS-PP22+AS-PP23) or siRNA (si-PP21, si-PP22, si-PP23) specific for PP2A-Cα. **A:** PP2A-Cα and PP1 levels were measured by immunoblot analysis at 3 days after transfection with antisense or missense oligos. **B & C:** Cells transfected with (B) or without (C) different antisense oligos or with missense oligo (○, None; ●, missense; ■, AS-PP22; ◆, AS-PP23; ▲, AS-PP22 + AS-PP23) were grown on Transwell inserts. TER was measured at varying times after administration of hydrogen peroxide (20 μM). Values are mean ± sem (n = 6). Asterisks indicate the values that are significantly ($P<0.05$) different from corresponding values for antisense groups. **D:** Cells transfected with or without antisense oligos or missense oligo were incubated with or without hydrogen peroxide. At 120 min after

hydrogen peroxide administration, inulin permeability was measured. Values are mean ± sem (n = 6). Asterisks indicate the values that are significantly ($p<0.05$) different from corresponding control values. Symbol # indicate the values that are significantly ($P<0.05$) different from corresponding values for None or Missense groups. **E:** Three days after transfection with different siRNA or control RNA cell extracts were immunoblotted for PP2A-Cα and PP1. **F:** At 2 hours after administration of hydrogen peroxide, inulin permeability was measured. Values are mean ± sem (n = 6). Asterisks indicate the values that are significantly ($p<0.05$) different from values for corresponding hydrogen peroxide free control group. Symbol # indicate the values different from values for scrambled RNA. **G:** Caco-2 cell monolayers were treated with (●) or without (■) hydrogen peroxide. Phospho-Thr was immunoprecipitated (IP) from denatured protein extracts from missense control RNA or si-PP23 and immunoblotted (IB) for occludin.

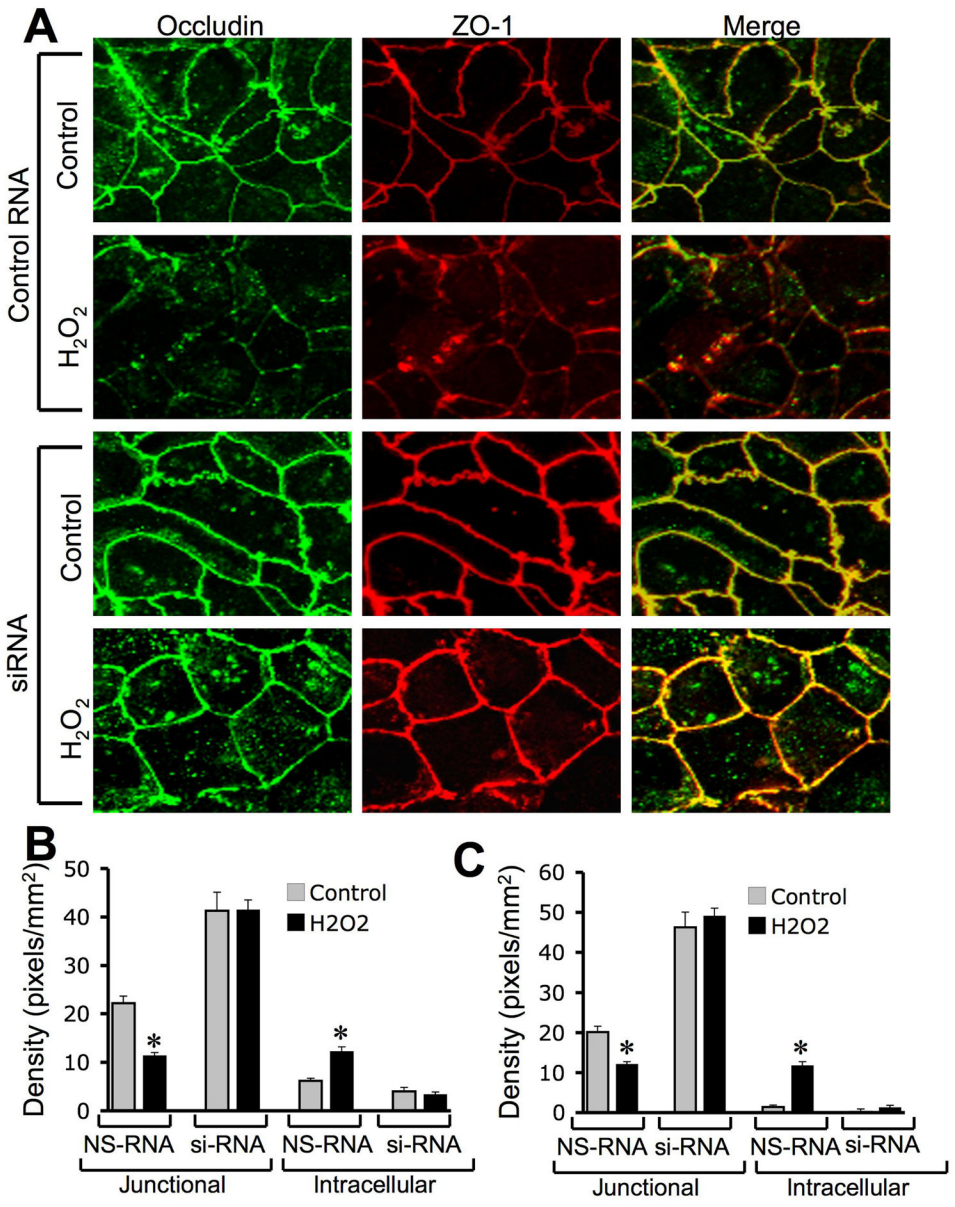

Fig. 5: Reduced expression of PP2A-Cα attenuates hydrogen peroxide-induced disruption of tight junctions.

A: Cells transfected with siRNA (si-PP23) or control RNA were grown on Transwell inserts. At 2 hour after administration of hydrogen peroxide (20 μM), cell monolayers were fixed and stained for occludin and ZO-1 by immunofluorescence method. Fluorescence images were collected by confocal microscopy. **B & C:** Intensity of occludin (B) and ZO-1 (C) fluorescence at the intercellular junctions and the intracellular compartments was measured by using the software Image J. Values are mean ± sem (n = 4). Asterisk indicates the value for hydrogen peroxide-treated cell monolayers that is significantly ($P<0.05$) different from corresponding value for control group.

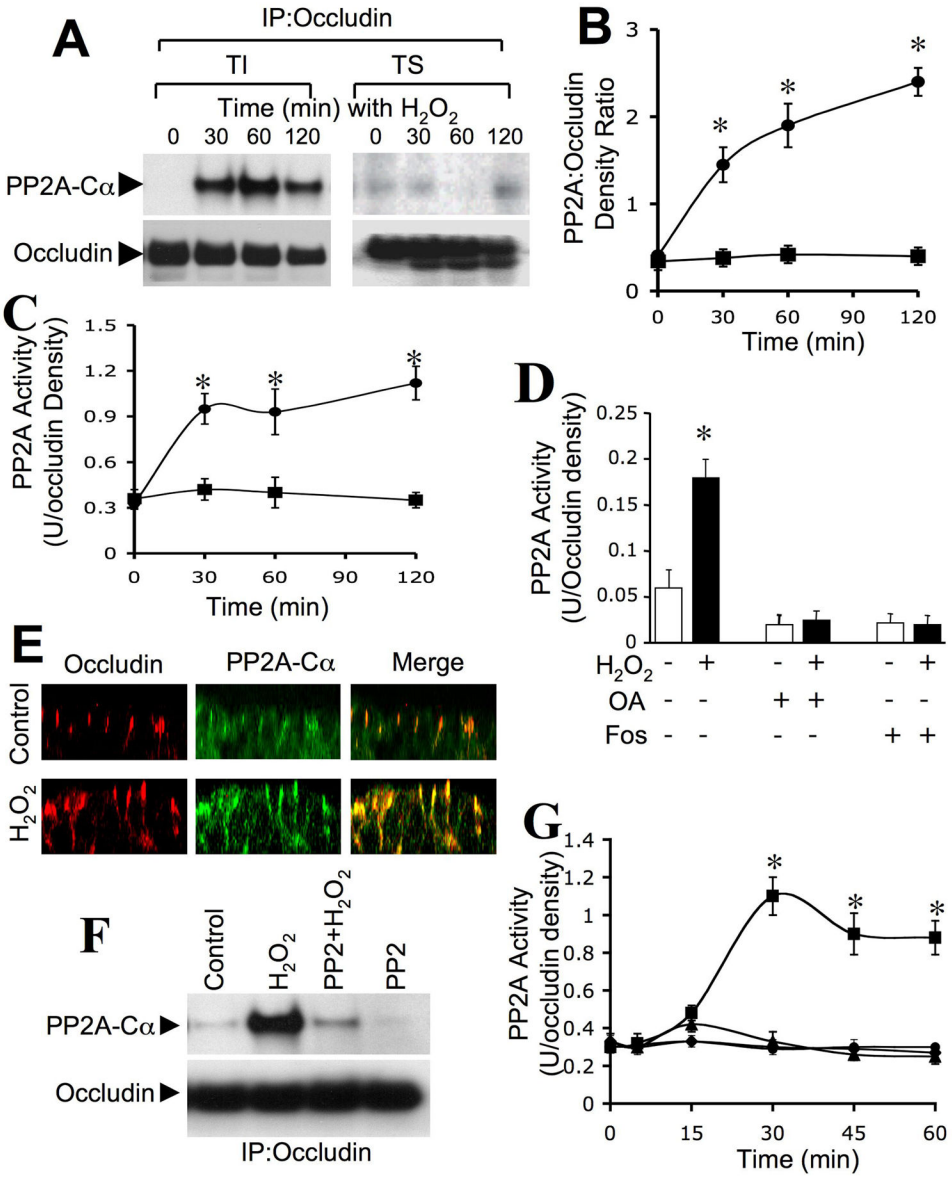

Fig. 6: hydrogen peroxide increases association of PP2A-Cα with occludin by a Src kinase dependent mechanism.

A: Caco-2 cell monolayers were incubated with hydrogen peroxide for varying times. Triton-insoluble (TI) and soluble (TS) fractions were prepared. Occludin was immunoprecipitated and the immunocomplexes were immunoblotted for PP2A-Cα and occludin. **B:** Anti-occludin immunocomplexes from cells treated with (●) or without (■) hydrogen peroxide were immunoblotted for PP2A-Cα. The densitometric values for PP2A bands are presented after normalization with corresponding densitometric values for occludin bands. Values are mean ± sem (n = 3). Asterisks indicate the values that are significantly ($p<0.05$) different from corresponding control values. **C:** Caco-2 cell monolayers were incubated with (●) or without (■) hydrogen peroxide for varying times. Occludin was immunoprecipitated from Triton-insoluble fractions and assayed for PP2A activity. Activity values were normalized to occludin band density. Values are mean ± sem (n

= 3). Asterisks indicate the values that are significantly ($p<0.05$) different from corresponding control values. **D:** Caco-2 cell monolayers pretreated with okadaic acid (OA) or fostreicin (Fos) were incubated with hydrogen peroxide for 2 hours. Occludin was immunoprecipitated from Triton-insoluble fractions and assayed for PP2A activity. Activities were normalized to occludin band density. Values are mean ± sem (n = 3). Asterisk indicates the value that is significantly ($p<0.05$) different from corresponding control value. **E:** Control and hydrogen peroxide-treated cell monolayers (60 min) were fixed and co-stained for occludin and PP2A-Cα. Flourescence images collected by confocal microscopy. **F:** Anti-occludin immunocomplexes from cells incubated with or without PP2 and with or without hydrogen peroxide were immunoblotted for PP2A-Cα and occludin. **G:** Caco-2 cell monolayers were pretreated with (◆, ■) or without (●, ▲) PP2 (Src kinase inhibitor) prior to incubation with (▲, ■) or without (●, ◆) hydrogen peroxide for varying times. PP2A activity was measured in anti-occludin immunocomplexes prepared from Triton-insoluble fractions. Values are mean ± sem (n = 3). Asterisks indicate the values that are significantly ($p<0.05$) different from corresponding values for control or PP2- hydrogen peroxide groups.

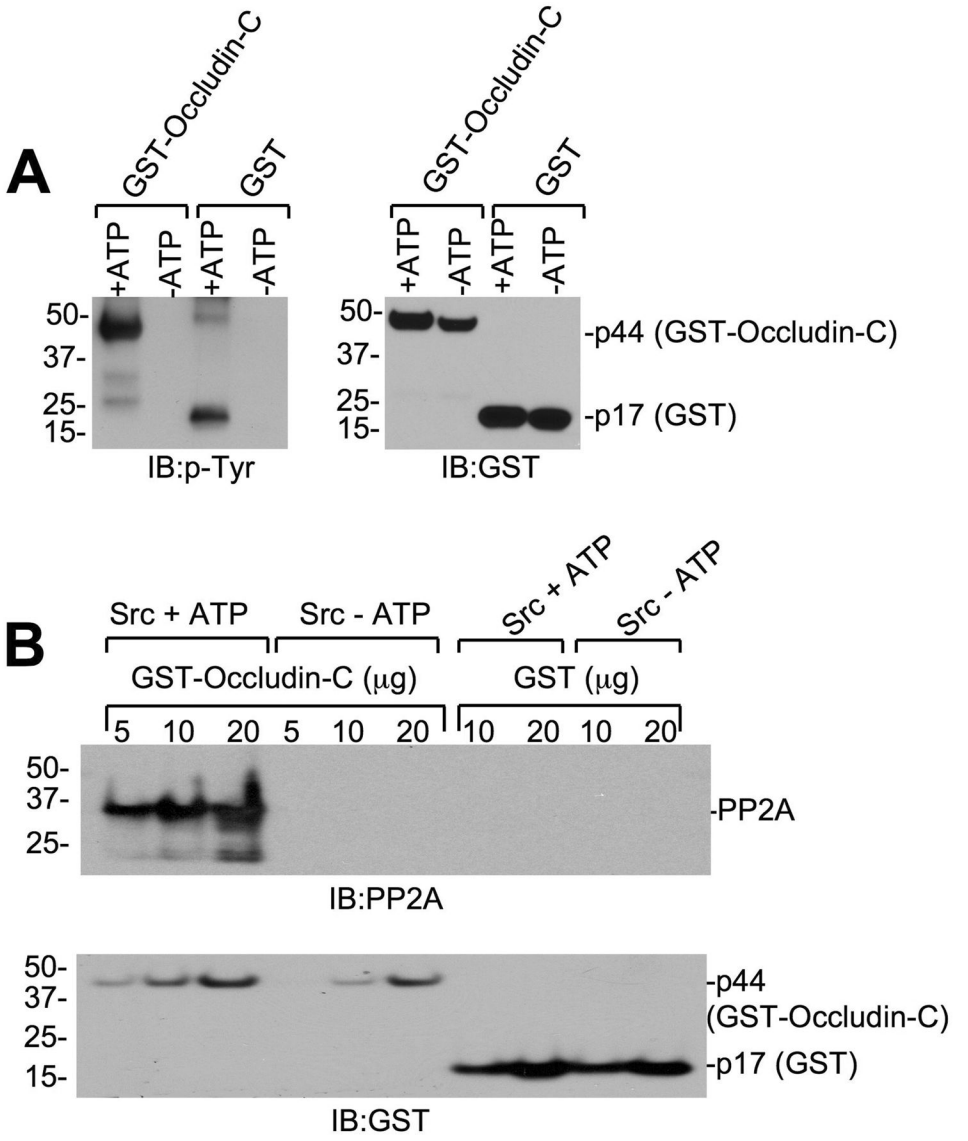

Fig. 7: PP2A binds to the C-terminal tail of occludin by phosphorylation-dependent mechanism.

A: GST-Occludin-C that was incubated with active c-Src in the absence or presence of ATP was immunoblotted for p-Tyr and GST. **B:** GST-fused C-terminal domain of occludin (GST-Occludin-C; 5–20 μg) was Tyr-phosphorylated by incubation with active c-Src in the presence of ATP. For non-phosphorylated controls, GST-Occludin-C was incubated with c-Src in the absence of ATP. Both phosphorylated and non-phosphorylated occludins were incubated with purified PP2A (50 ng). GST-Occludin-C was pulled down with GSH-agarose and immunoblotted for PP2A-Cα. PP2A binding to GST was performed as a control. The blot was reprobed for GST to locate and evaluate GST-Occludin-C and GST. The numbers on the left side of all blots represent the location and molecular weight of standard proteins.