Expression of Kinase-inactive c-Src Delays Oxidative Stress-induced Disassembly and Accelerates Calcium-mediated Reassembly of Tight Junctions in the Caco-2 Cell Monolayer*

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The activity of Src kinases appears to play a role in both assembly and disassembly of tight junction. However, the role of a specific isoform of Src kinase in regulation of tight junction is not known. In the present study the role of c-Src in regulation of epithelial tight junction was investigated in Caco-2 cell monolayers. Oxidative stress (xanthine oxidase + xanthine) induced an activation and membrane translocation of c-Src. The oxidative stress-induced decrease in transepithelial electrical resistance, increase in inulin permeability, and redistribution of occludin and ZO-1 from the intercellular junctions were prevented by PP2. The rates of oxidative stress-induced activation of c-Src, tyrosine phosphorylation of ZO-1 and β-catenin, decrease in resistance, increase in permeability to inulin, and redistribution of occludin and ZO-1 were significantly greater in cells transfected with wild type c-Src, whereas it was low in cells transfected with kinase-inactive c-SrcK297R mutant, when compared with those in empty vector-transfected cells. The rates of recovery of resistance, increase in barrier to inulin, and reorganization of occludin and ZO-1 into the intercellular junctions during the calcium-induced reassembly of tight junction were much greater in Caco-2 cells transfected with c-SrcK297R as compared with those in cells transfected with empty vector or wild type c-Src. These results show that the dominant-negative expression of kinase-inactive c-Src delays the oxidative stress-induced disruption of tight junction and accelerates calcium-induced assembly of tight junction in Caco-2 cells and demonstrate that oxidative stress-induced disruption of tight junction is mediated by the activation of c-Src.

The tight junction (TJ) forms a barrier to the diffusion of toxins, allergens, and pathogens across the epithelial tissue.
Regulation of Epithelial Tight Junction by c-Src

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In the present data demonstrate that c-Src is involved in the de-stabilization of TJ. We transfected Caco-2 cells with empty expression vector, pUSEc-Src (wild type), and pUSEc-SrcK297R (kinase-inactive mutant) to determine the role of c-Src in regulation of the epithelial TJ. We show that 1) oxidative stress induces activation and membrane translocation of c-Src in Caco-2 cells, 2) oxidative stress-induced increase in paracellular permeability is prevented by PP2, the inhibitor of Src family kinases, 3) expression of kinase-inactive c-Src (c-SrcK297R mutant) delays oxidative stress-induced TJ permeability, tyrosine phosphorylation of ZO-1 and β-catenin, and redistribution of occludin and ZO-1, whereas the expression of wild type c-Src induces slight potentiation of the effect of oxidative stress, and 4) expression of c-SrcK297R accelerates the assembly of TJ during the recovery after calcium switch.

EXPERIMENTAL PROCEDURES

Chemicals—Cell culture reagents including LipofectAMINE and Genetin were purchased from Invitrogen. EGTA, xanthine oxidase, xanthine, streptavidin-agarose, fluorescein isothiocyanate-conjugated inhibitor, protease inhibitors, and protein-A-Sepharose were purchased from Sigma. PP2 (AG1879, 4-amino-5-chlorophenyl)-7-(6-butyryl)pyrazolo[5-4-d]pyrimidine) was purchased from Calbiochem. All other chemicals were of analytical grade purchased either from Sigma or Fisher. Preset SDS-polyacrylamide gels were purchased from Invitrogen.

Antibodies—Mouse monoclonal anti-occludin and rabbit polyclonal anti-occludin and anti-ZO-1 antibodies were purchased from Zymed Laboratories Inc. (South San Francisco, CA). Biotin-conjugated anti-phosphotyrosine and Cy3-conjugated goat anti-rabbit IgG were purchased from Sigma. Peroxidase-conjugated anti-phosphotyrosine antibodies were from Chemicon. Alexa-fluor® 568-conjugated anti-mouse IgG and Cy-3-conjugated anti-rabbit IgG. The fluorescence was analyzed as described before (19) by confocal laser-scanning microscopy (Bio-Rad MRC1024). Images were processed using the software Confo- cal Assistant 4.02 and Adobe Photoshop (Adobe Systems Inc., San Jose, CA).

Preparation of Plasma Membrane Fraction—Caco-2(EV), Caco-2(WT), and Caco-2(KI) cell monolayers (24-mm Transwell) were washed twice with ice-cold PBS and once with lysis buffer-F (PBS containing 10 mM β-glycerophosphate, 2 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml bestatin, 10 μg/ml pepstatin-A, and 1 μg/ml benzamidine). Total 1 ml phenylmethylsulfonyl fluoride (PMSF) was added before lysis and homogenized as described before (19). The plasma membrane pellet was suspended in 500 μl of lysis buffer F. Protein was measured by the BCA method (Pierce). Membrane fraction was either mixed with an equal volume of Laemmli sample buffer (2× concentrated) and heated at 100 °C for 5 min or dissolved in lysis buffer N (20 mM Tris, pH 7.4, containing 0.02% Nonidet P-40, 0.1% sodium deoxycholate, and a mixture of protease inhibitors as described above for lysis buffer-F) for immunoprecipitation of c-Src.

Immunoprecipitation—After XO + X treatment, Caco-2(EV), Caco-2(WT), and Caco-2(KI) cell monolayers (24 mm) were washed with ice-cold 20 mM Tris, pH 7.4, and the proteins were extracted in lysis buffer-N containing 0.5% SDS in 10 mM Tris buffer, pH 7.4, containing 1 mM vanadate and 0.33 mM phenylmethylsulfonyl fluoride) under denaturing conditions (heating at 100 °C for 5 min). Phosphotyrosine was immunoprecipitated as described before (19) using biotin-conjugated anti-phosphotyrosine antibody. Immune complexes were isolated by precipitation using streptavidin-agarose. Immunoprecipitates were immunoblotted for ZO-1 and β-catenin using rabbit polyclonal anti-ZO-1 and mouse monoclonal anti-β-catenin antibodies, respectively. For immu- nocomplex c-Src kinase assay, proteins from plasma membrane fraction were extracted under native conditions with lysis buffer N. c-Src was immunoprecipitated by using mouse monoclonal anti c-Src antibody and proteins were analyzed by SDS-polyacrylamide gel (4–12% gradient) electrophoresis and electroblotted into polyvinylidene difluoride membranes. Membranes were probed for ZO-1 or β-catenin, or c-Src, or pTyr(Sp)418 using rabbit polyclonal anti-ZO-1 and anti-c-Src(Y418) and mouse monoclonal anti-c-Src and anti-β-catenin antibodies in combination with horseradish peroxidase-conjugated anti-phosphotyrosine antibodies (made in our laboratory) or peroxidase-conjugated anti-rabbit IgG antibodies. Blots were developed using ECL chemiluminescence kit (Amersham Biosciences).

Tyrosine Kinase Assay—Plasma membrane and soluble fractions or anti-c-Src immune complexes were incubated in an assay mixture containing 50 mM imidazole, pH 7.4, 150 mM NaCl, 2 mM MgCl₂, 12 mM HEPES, 1.7 mM ATP, 0.1 mM ß-glycerophosphate, 20 μM PMSF, 0.1% Triton X-100, 5 μg of poly-(Glu-Tyr) peptide substrate, and 1 μCi of [γ-32P]ATP at 25 °C for 30 min. After incubation proteins were precipitated with an equal volume of 5% trichloroacetic acid, and a 50-μl aliquot of trichloroacetic acid-soluble fraction was treated cell monolayers were then incubated in regular DMEM without EGTA for 4–8 h under standard cell culture conditions. Reassembly of TJ and restoration of barrier function were assessed at varying times by measuring the transepithelial electrical resistance (TER) andulin flux.

Measurement of TER—TER was measured as described previously (15) using a Millicell-ERS Electrical Resistance System (Millipore, Bedford, MA). TER was calculated as ohms/cm² by multiplying it with the surface area of the monolayer (0.33 cm²). The resistance of the supporting membrane in Transwells (which is usually around 30 ohms/cm²) was subtracted from all readings before calculations.

Unidirectional Flux of Inulin—Cell monolayers in Transwells were incubated under different experimental conditions in the presence of 5 μCi/ml 125I-conjugated inulin (specific activity 100 mCi/mmol). At varying times after experimental treatments, 150 μl of apical medium and 50 μl of basal medium were withdrawn. Fluorescence was measured in a FLX 800 microplate fluorescence reader (BioTEK Instruments Inc, Winooski, VT). The flux into the apical well was calculated as the percent of total inulin administered into the basal well/μl per cm² of surface area.

Immunofluorescence Microscopy—Under different experimental conditions, Caco-2(EV), Caco-2(WT), and Caco-2(KI) cell monolayers were washed in PBS, fixed in acetone: methanol (1:1), and blocked in 20 mM Tris, pH 7.2, 150 mM NaCl, and 1% bovine serum albumin as described before (19). Cell monolayers were incubated with primary antibodies (anti-ZO-1 and anti-c-Src) for 1 h followed by secondary antibodies (Alexa-fluor® 568-conjugated anti-mouse IgG and Cy3-conjugated anti-rabbit IgG). The fluorescence was visualized using a Leica fluorescent microscope (Bio-Rad MRC1024). Images were processed using the software Confo- cal Assistant 4.02 and Adobe Photoshop (Adobe Systems Inc., San Jose, CA).
blotted on to P81 Whatman filter discs. Discs were washed in 0.5% phosphoric acid. Radioactivity in air-dried discs was counted in a scintillation counter, Beckman LS500TA (Beckman Coulter, Inc., Fullerton, CA). Activity was calculated as units of pmol of phosphate incorporated to substrate/h and presented as units/mg of protein in plasma membrane or soluble fractions or protein used for immunoprecipitation of c-Src. Activity present in corresponding immune complexes prepared under denaturing conditions. Phosphotyrosine (PY) and c-Src were immunoprecipitated (IP) followed by immunoblot (IB) analysis for c-Src and phosphotyrosine, respectively. D, whole protein extracts prepared from cell monolayers treated with XO + X for varying times were immunoblotted for total c-Src and active c-Src[PY418] using specific antibodies.

**RESULTS**

**Oxidative Stress Induces Activation and Membrane Translocation of c-Src**—The activation of c-Src by oxidative stress in Caco-2 cells was determined by measuring the tyrosine kinase activity, assessing the phosphorylation of c-Src on tyrosine 418, and the translocation of c-Src into plasma membranes. Treatment with XO + X resulted in a rapid increase in overall tyrosine kinase activity in the plasma membrane fraction, which was associated with a decrease in tyrosine kinase activity in the soluble fraction (Fig. 1A). Immune complex tyrosine kinase assay showed that the activity of c-Src in the plasma membrane was also rapidly increased by XO + X (Fig. 1B). Immunoprecipitation of phosphotyrosine followed by immunoblot analysis for c-Src showed that XO + X treatment resulted in a rapid increase in tyrosine phosphorylation of c-Src in Caco-2 cells (Fig. 1C). Immunoblot analysis also shows that XO + X induced a rapid increase in the phosphorylation of c-Src on tyrosine 418 (Fig. 1D), whereas the level of total c-Src was unaffected. The level of c-Src in the plasma membrane was rapidly increased by XO + X treatment (Fig. 2A), which was associated with a concomitant decrease in c-Src in the soluble fraction. The level of phosphorylation of c-Src on tyrosine 418 was increased in both plasma membrane and soluble fractions (Fig. 2B).

The Activity of Src Family Kinases Is Involved in Oxidative Stress-induced Disruption of TJ—Our previous studies demonstrated that tyrosine kinase activity plays a role in the oxidative stress-induced increase in TJ permeability (15–19). The role of Src family kinase activity in oxidative stress-induced TJ permeability was determined by evaluating the effect of PP2, a selective inhibitor of Src family kinases. Administration of PP2 significantly blocked the XO + X-induced decrease in TER (Fig. 3A) and increase in inulin permeability (Fig. 3B). PP2 also prevented XO + X-induced redistribution of occludin and ZO-1 from the intercellular junctions (Fig. 3C). Cell viability was evaluated and compared between groups by several methods, such as lactate dehydrogenase release, DNA fragmentation, trypan blue exclusion, and nuclear staining with propidium iodide. All methods showed that there was less than 0.03% cell death, and these values were similar for non-treated and XO + X-treated cell monolayers.

Expression of Wild Type c-Src and Kinase-inactive c-Src Mutant in Caco-2 Cells—Caco-2 subclones, Caco-2(WT), and Caco-2(KI), expressing the empty vector, wild type c-Src, and kinase-inactive c-SrcK297R mutant, respectively, were isolated. Immunoblot analysis shows an overexpression of c-Src in Caco-2(WT) and Caco-2(KI) cells compared with c-Src expression in Caco-2(EV) cells (Fig. 4A). The gross morphology of these clones in culture was not very different from one another, except that Caco-2(KI) cells grew with slightly greater density (8–12% more cells per unit area) than the others (data not shown). TER tends to be slightly low in Caco-2(WT) cell monolayer and high in Caco-2(KI) cell monolayer compared with the TER in Caco-2(EV) cell monolayer (Fig. 4B). Similarly, inulin permeability tends to be slightly high in Caco-2(WT) cell monolayer and low in Caco-2(KI) cell monolayer compared with that in Caco-2(EV) cell monolayer (Fig. 4C). The differences
between Caco-2(WT) and Caco-2(KI) were statistically significant.

Expression of Kinase Inactive c-Src(K297R) Mutant Delays Oxidative Stress-induced Increase in Paracellular Permeability——Four distinct clones each from Caco-2(EV), Caco-2(WT), and Caco-2(KI) were analyzed. Treatment with XO + X resulted in a time-dependent decrease in TER in Caco-2(EV) cell monolayers (Fig. 5A). The rates of decrease in TER by XO + X were significantly low in Caco-2(KI) cell monolayers, whereas those in Caco-2(WT) cell monolayers were greater as compared with Caco-2(EV) cell monolayers. At 1 h of XO + X treatment, the TERs for Caco-2(EV), Caco-2(WT), and Caco-2(KI) monolayers were 73 ± 5, 38 ± 3, and 108 ± 8% of corresponding basal values, respectively. Similarly, inulin permeability measured at 2 h of oxidative stress was significantly low in Caco-2(KI) cell monolayers, whereas it was greater in Caco-2(WT) cell monolayers as compared with inulin permeability in Caco-2(EV) cell monolayers (Fig. 5B).

Expression of Kinase Inactive c-Src Mutant Reduces Oxidative Stress-induced Activation of c-Src——Studies described above show that oxidative stress induces activation and membrane translocation of c-Src in Caco-2 cell monolayers. To determine a similar activation of c-Src in different subclones,
plasma membrane and soluble fractions were isolated from the control and the XO + X-treated Caco-2(EV), Caco-2(WT), and Caco-2(KI) cell monolayers. Distribution of total and active c-Src[pY418] in the plasma membrane fraction was determined by immunoblot analysis using anti-Src and anti-c-Src[pY418] antibodies, respectively. Total amount of c-Src present in plasma membranes was increased by XO + X treatment in all subclones (Fig. 7A). Active c-Src was not detectable in the plasma membrane of control cell monolayers, whereas it was present in XO + X-treated cell monolayers (Fig. 7B). However, the amount of c-Src[pY418] present in the plasma membranes of Caco-2(KI) cells was much less than the amounts of c-Src[pY418] present in the membranes from Caco-2(EV) and Caco-2(WT) cells. We also measured the c-Src kinase activity in the plasma membranes by immune complex tyrosine kinase activity. Very low levels of c-Src kinase activity were detected in the plasma membranes of untreated Caco-2(EV), Caco-2(WT), and Caco-2(KI) cell monolayers, whereas the activity was increased by XO + X treatment (Fig. 7C). However, c-Src kinase activity in XO + X-treated Caco-2(KI) cells was significantly less than that in XO + X-treated Caco-2(EV) and Caco-2(WT) cells. The activity in XO + X-treated Caco-2 (WT) cells was slightly greater than that in Caco-2(WT) cells.

Oxidative Stress Induces Tyrosine Phosphorylation of ZO-1 and β-Catenin—Our previous study demonstrated that oxidative stress increases tyrosine phosphorylation of junctional proteins, such as ZO-1 and β-catenin, which appear to be important in the disassembly of TJ (19). Immunoprecipitation of phosphotyrosine from denatured extracts of cell monolayers followed by immunoblot analysis for ZO-1 and β-catenin detected no tyrosine-phosphorylated ZO-1 and β-catenin in untreated Caco-2(EV), Caco-2(WT), and Caco-2(KI) cell monolayers (Fig. 8). XO + X treatment resulted in the appearance of tyrosine-phosphorylated ZO-1 and β-catenin. The amount of tyrosine-phosphorylated ZO-1 and β-catenin was found to be less in XO + X-treated Caco-2(KI) cells, whereas it was greater in Caco-2(WT) cells compared with that in XO + X-treated Caco-2(EV) cell monolayers.

Expression of Kinase-inactive c-Src(K297R) Mutant Accelerates Calcium-mediated Assembly of TJ—To determine the role of c-Src in the assembly of TJ we evaluated the rate of recovery of barrier function after the disruption of TJ by calcium depletion. Treatment with EGTA for 20 min rapidly reduced the TER of Caco-2 (EV), Caco-2 (WT), and Caco-2 (KI) cell monolayers to around 70 ohms/cm². Replacement of calcium resulted in a time-dependent increase in TER in all cell monolayers (Fig. 9A). However, the rates of increase in TER in Caco-2 (KI) cell monolayers were dramatically high compared with those in Caco-2 (EV) cell monolayers, whereas those in Caco-2(WT) cell monolayers were significantly lower than those in Caco-2(EV) cell monolayers. After 4 h of recovery the TER was found to be 192 ± 18, 125 ± 3, and 421 ± 33 ohms/cm² in Caco-2(EV), Caco-2(WT), and Caco-2(KI) cell monolayers, respectively. Similarly, inulin permeability measured after 3 h of recovery was significantly low in Caco-2(KI) cell monolayers, whereas it was high in Caco-2(WT) cell monolayers as compared with that in
Caco-2(EV) cell monolayers (Fig. 9B). Immunofluorescence staining and confocal microscopy showed a redistribution of occludin (Fig. 10A) and ZO-1 (Fig. 10B) from the intercellular junctions of Caco-2(EV), Caco-2(WT), and Caco-2(KI) cell monolayers into the intracellular compartments by EGTA treatment. After 3 h of recovery only minimal amounts of occludin and ZO-1 were reorganized at the intercellular junctions in Caco-2(EV) and Caco-2(WT) cell monolayers, whereas major portions of occludin and ZO-1 were reorganized at the intercellular junctions of Caco-2(KI) cell monolayers.

The Activity of Src Family Kinases Is Involved in Oxidative Stress-induced Disruption of TJ in MDCK Cell Monolayer—
The above studies show that c-Src is required for the oxidative stress-induced disruption of TJ in the Caco-2 cell monolayer. Interestingly, this observation contrasts the previous observation by Meyer et al. (23) that Src kinase activity is required for the assembly of TJ in MDCK cell monolayers. To determine whether there is a difference in the mechanism of oxidative stress-induced disruption of TJ between Caco-2 and MDCK cell monolayers, the effect of XO + X in the absence or presence of PP2 on TJ permeability was evaluated. Administration of XO + X induced a decrease in TER (Fig. 11A), an increase in inulin permeability (Fig. 11B), and redistribution of occludin (Fig. 11C). PP2 significantly blocked the XO + X-induced changes in TER, inulin flux, and redistribution of occludin.

Fig. 7. XO + X-induced membrane translocation and activation of c-Src. Caco-2(EV), Caco-2(WT), and Caco-2(KI) cell monolayers were incubated with PBS or PBS containing XO + X for 3 h. After treatment, plasma membranes were isolated and analyzed by immunoblotting for total c-Src (A) and active c-Src[pY418] (B) as described under “Experimental Procedures” using mouse monoclonal anti-c-Src and rabbit polyclonal anti-c-Src[pY418] antibodies. c-Src kinase activity (C) was measured by immune complex c-Src kinase assay; values are the mean ± S.E. (n = 3), and asterisks indicate values that are statistically different from the values for Caco-2(EV) cells. U, units.

Fig. 8. XO + X-induced tyrosine phosphorylation ZO-1 and β-catenin. Caco-2(EV), Caco-2(WT), and Caco-2(KI) cell monolayers were incubated in the absence or presence XO + X for 3 h. Phosphotyrosine (PY) from proteins extracted under denaturing conditions were immunoprecipitated using biotin-conjugated anti-phosphotyrosine antibody. Immunoprecipitates (IP) were then analyzed for ZO-1and β-catenin by immunoblot (IB) analysis using rabbit polyclonal antizO-1 and mouse monoclonal anti-β-catenin antibodies.

stress-induced disruption of TJ in MDCK cell monolayers, the effect of XO + X in the absence or presence of PP2 on TJ permeability was evaluated. Administration of XO + X induced a decrease in TER (Fig. 11A), an increase in inulin permeability (Fig. 11B), and redistribution of occludin (Fig. 11C). PP2 significantly blocked the XO + X-induced changes in TER, inulin flux, and redistribution of occludin.

DISCUSSION

The proto-oncogene, c-Src, is a non-receptor tyrosine kinase that plays a crucial role in a number of physiological functions of normal cells, such as cell proliferation, differentiation, and cell migration (37). c-Src is transiently activated during growth factor receptor activation and mitosis (38), whereas prolonged activation of c-Src due to molecular modifications or mutation leads to cell transformation. Elevations of c-Src kinase activity have been altered in a variety of human cancers, including colon cancer (39, 40). c-Src tyrosine phosphorylates a wide variety of cellular proteins, including platelet-derived growth factor, epidermal growth factor, and CSF-1 receptors, focal adhesion kinase, proline-rich tyrosine kinase-2, Cdk-activating kinase, vinculin, and lactate dehydrogenase (41). Localization of c-Src at the TJ of epithelial tissue suggests that Src kinase activity may play an important role in the regulation of TJ structure and function (1). Our recent studies indicated that...
tyrosine kinase activity and protein tyrosine phosphorylation play an important role in the regulation of TJ in the Caco-2 cell monolayer (15–19). These studies raised the issue of whether c-Src plays a role in the regulation of TJ under basal condition or under oxidative stress. Here we show that overexpression of kinase-inactive c-Src mutant delays oxidative stress-induced increase in paracellular permeability and accelerates calcium-mediated assembly of TJ.

Inhibition of XO + X-induced decrease in TER, increase in inulin permeability, and redistribution of occludin and ZO-1 from the intercellular junctions by PP2, a Src family kinase inhibitor, indicate that the activity of Src family kinases is involved in the oxidative stress-induced disruption of TJ and increase in paracellular permeability. A previous study showed a similar inhibition of hydrogen peroxide-induced paracellular permeability in an endothelial cell monolayer (34); however, redistribution of TJ proteins or activation of Src family kinases were not analyzed. Our present data show that the tyrosine kinase activity of c-Src in the plasma membrane was strongly increased by XO + X treatment. Additionally, treatment with XO + X induced tyrosine phosphorylation of c-Src, particularly phosphorylation on tyrosine 418, and translocation of c-Src protein into the plasma membrane. Tyrosine 418 is an auto-phosphorylation site, and therefore, the increase in phospho-rylation at this site is an indicator of the activity of c-Src. These results clearly demonstrate that oxidative stress rapidly activates c-Src in the Caco-2 cell monolayer and raises the issue of whether c-Src activity plays a role in the disruption of TJ. However, there is a significant interval between the activation of c-Src and the decrease in TER. It is quite likely that a rapid activation of c-Src sets the stage for subsequent events that lead to the opening of TJ. Evidence indicates that disruption of TJ by oxidative stress involves protein kinase C activity, dephosphorylation of occludin on serine and threonine residues, and reorganization of actin cytoskeleton. These may well be some of the intermediate steps involved in TJ disruption by oxidative stress.

To determine the role of c-Src in regulation of TJ, we transfected Caco-2 cells with the wild type c-Src or kinase-inactive c-SrcK297R mutant. The overexpression of wild type c-Src and kinase-inactive c-SrcK297R mutant was determined by the increased levels of total c-Src present in the extracts of Caco-2(EV), Caco-2(WT), and Caco-2(KI) subclones. c-Src levels in Caco-2(WT) and Caco-2(KI) cells were greater than that in Caco-2(EV) cells. Although the gross morphology of different clones was similar, there was a tendency for Caco-2(KI) cells to grow more densely than Caco-2(EV) and Caco-2(WT) cells. The paracellular permeability tends to be slightly low in Caco-2(KI-Src) cell monolayers and greater in the Caco-2(WT-Src) cell monolayer compared with that in Caco-2(EV) cell monolayers, although these differences were not statistically significant.

To determine the role of c-Src in the oxidative stress-induced disruption of TJ, we evaluated the effect of XO + X on paracellular permeability and redistribution of TJ proteins in Caco-2(EV), Caco-2(WT), and Caco-2(KI) cell monolayers. The rate of oxidative stress-induced increase in permeability was slightly greater in Caco-2(WT-Src) cell monolayers, whereas it was dramatically delayed in Caco-2(KI) cell monolayers when compared

Fig. 10. Immunofluorescence localization of occludin during reassembly of TJ. Caco-2(EV), Caco-2(WT), and Caco-2(KI) cell monolayers were incubated for 20 min with EGTA followed by washing and replacement of calcium. At 0 and 3 h of recovery, cell monolayers were fixed and labeled for occludin (A) and ZO-1 (B) by immunofluorescence staining as described under “Experimental Procedures” using mouse monoclonal anti-occludin and rabbit polyclonal anti-ZO-1 antibodies. Images were collected by confocal microscopy.

Fig. 11. Activity of Src family kinases is involved in oxidative stress-induced disruption of tight junction in MDCK cell monolayers. MDCK cell monolayers were incubated with PBS (■) and PBS containing XO + X in the absence (▲) or presence (●) of PP2 (10 μM) for varying lengths of time. TER (A) was measured at varying times, whereas inulin flux (B) was measured after 3 h of incubations. Values are mean ± S.E. (n = 6). Cell monolayers incubated for 3 h with XO + X without or with PP2 were fixed and labeled for occludin by immunofluorescence staining (C).
with that in Caco-2(EV) cell monolayers. This observation supports the view that activation of c-Src plays an important role in oxidative stress-induced disassembly of TJ. It was further supported by the confocal immunofluorescence microscopic observation that oxidative stress causes a reorganization of TJ proteins occludin and ZO-1 in Caco-2(EV) and Caco-2(WT) cell monolayers, whereas it is relatively unaffected in Caco-2(KI) cell monolayers.

Oxidative stress induced a translocation of c-Src to the plasma membranes and increased the level of active c-Src[pY418] in the plasma membranes on Caco-2(EV) cells. The membrane translocation of total c-Src was found to be similar in Caco-2(EV), Caco-2(WT), and Caco-2(KI) cells. However, the level of active c-Src[pY418] present in the plasma membrane was different from each other. The amount of active c-Src[pY418] present in plasma membrane of XO + X-treated Caco-2(WT) cells was slightly greater than that in membranes of XO + X-treated Caco-2(EV) cells, whereas the amount of active c-Src[pY418] in plasma membranes of XO + X-treated Caco-2(KI) cells was much lower compared with those in XO + X-treated Caco-2(EV) and Caco-2(WT) cells. These results indicate that translocation of total amount of c-Src to plasma membrane was unaffected by the overexpression of wild type c-Src or kinase-inactive c-Src[K297R]. However, translocation of inactive c-Src[K297R] may have resulted in the presence of low levels of active c-Src[pY418] in the plasma membrane of XO + X-treated Caco-2(KI) cell monolayers. This observation was further supported by similar differences in the c-Src tyrosine kinase activity in the plasma membranes of Caco-2(EV) Caco-2(WT), and Caco-2(KI) cells. Furthermore, the extent of tyrosine phosphorylation of ZO-1 and β-catenin in XO + X-treated Caco-2(KI) cell monolayer was lower compared with that in XO + X-treated Caco-2(EV) and Caco-2(WT) cell monolayers. ZO-1 and β-catenin tyrosine phosphorylation was greater in XO + X-treated Caco-2(WT) cells. Our recent study demonstrated that oxidative stress induces tyrosine phosphorylation of proteins of TJ and adherens junction, in particular tyrosine phosphorylation of ZO-1 and β-catenin associated with the actin cytoskeleton (19). These results demonstrate that the oxidative stress-induced c-Src activation in the plasma membrane and tyrosine phosphorylation of ZO-1 and β-catenin were attenuated by the dominant negative expression of kinase-inactive c-Src in Caco-2 cells.

The outcome of this study that the tyrosine kinase activity of c-Src is involved in the disassembly of TJ in the canine kidney epithelium is in contrast to the previous reports that Src family kinase activity is required for the assembly of TJ in the canine kidney epithelium (23). There are two likely explanations for such a discrepancy in the role of Src kinase activity in the regulation of TJ. First, the possibility exists that there is a cell-type-dependent difference in signaling pathways and their role in regulation of TJ. Second, it is quite likely that Src kinase activity is required for both the assembly and disassembly of TJ; different members of Src family kinase may be involved in regulation of TJ. To determine whether there is a cell-type dependent difference in the mechanism of XO + X-induced increase in TJ permeability, we evaluated the effect of XO + X in the absence and presence of PP2. The results showed that XO + X induces a decrease in TER, an increase in inulin permeability, and redistribution of occludin in MDCK cell monolayers. These XO + X-induced changes in TER, inulin flux, and redistribution of occludin were prevented by PP2. This observation in the MDCK cell monolayer is similar to the observation made in Caco-2 cells, suggesting that the mechanism of TJ regulation is similar in both Caco-2 and MDCK cell monolayers. However, the new observation in combination with a previous study by Meyer et al. (23) demonstrates that Src activity is required for both the disruption and the assembly of TJ, suggesting that distinct members of Src family kinases may be involved in the assembly and the disruption of TJ. As shown by our present data c-Src activity may regulate the disassembly of TJ, whereas other Src kinases such as c-Yes may be involved in the assembly of TJ. C-Yes appears to bind occludin C-terminal peptide (OCT) and play a role in the assembly of TJ in MDCK cell monolayers (21).

The present study also shows that the expression of wild type and kinase-inactive c-Src alters the ability of Caco-2 cells to reassemble TJ after the disruption by calcium depletion. As shown previously in MDCK cell monolayers (42), calcium depletion resulted in a rapid disruption of the TJ and increase in paracellular permeability in Caco-2(EV) cell monolayer. Replacement of calcium resulted in a gradual reassembly of TJ and restoration of paracellular barrier function. Interestingly enough, restoration of barrier function was delayed in Caco-2(WT) cells, whereas it was dramatically accelerated in Caco-2(KI) cells. This difference in the rate of restoration of barrier function in Caco-2(EV), Caco-2(WT), and Caco-2(KI) cells was supported by immunofluorescence localization of the TJ proteins occludin and ZO-1. After 3 h of recovery, occludin and ZO-1 remained disorganized in the intracellular compartments of Caco-2(EV) and Caco-2(WT), whereas occludin and ZO-1 were reorganized at the intercellular junctions of Caco-2(KI) cell monolayer. These data indicate that the expression of wild type c-Src delays the assembly of TJ, whereas the expression of kinase-inactive c-Src[K297R] accelerates the assembly of TJ and restoration of the barrier function.

In summary, this study shows that c-Src tyrosine kinase activity delays the calcium-mediated assembly and accelerates the oxidative stress-induced disassembly of TJ in Caco-2 cell monolayers. Results also indicate that activation of c-Src and its activity play a crucial role in the oxidative stress-induced disruption of TJ and increase in paracellular permeability.

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