# Tyrosine phosphorylation and dissociation of occludin–ZO-1 and E-cadherin–$\beta$-catenin complexes from the cytoskeleton by oxidative stress

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The oxidative-stress-induced alteration in paracellular junctional complexes was analysed in Caco-2 cell monolayer. Oxidative stress induced a rapid increase in tyrosine phosphorylation of occludin, zonula occludens (ZO)-1, E-cadherin and $\beta$-catenin. An oxidative-stress-induced decrease in transepithelial electrical resistance was associated with a redistribution of occludin–ZO-1 and E-cadherin–$\beta$-catenin complexes from the intercellular junctions. Genistein, a tyrosine kinase inhibitor, prevented the oxidative-stress-induced decrease in resistance and redistribution of protein complexes. Occludin, ZO-1, E-cadherin and $\beta$-catenin in the Triton-insoluble cytoskeletal fraction were reduced by oxidative stress, which was prevented by genistein. Oxidative stress also reduced the co-immunoprecipitation of ZO-1 with occludin, which was prevented by genistein. Co-immunoprecipitation of $\beta$-catenin with E-cadherin was unaffected by oxidative stress or genistein. ZO-1, E-cadherin and $\beta$-catenin in the plasma membrane or membrane-cytoskeleton were either slightly reduced or unaffected by oxidative stress or genistein. These results show that oxidative stress induces tyrosine phosphorylation and cellular redistribution of occludin–ZO-1 and E-cadherin–$\beta$-catenin complexes by a tyrosine-kinase-dependent mechanism.

Key words: cell–cell adhesion, epithelium, paracellular permeability, tight junction, tyrosine kinase.

## INTRODUCTION

The tight junctions (TJs) and the adherens junctions (AJs) form the barrier to the diffusion of macromolecules across the epithelium in different tissues. Whereas TJs form a physical barrier to the diffusion of macromolecules through the paracellular space, AJs may indirectly regulate the structure and function of TJs. Studies during the past decade have identified a number of specific proteins localized at the TJ and AJ. Occludin [1], claudins [2] and junction adhesion molecule [3] are the transmembrane proteins currently known to be localized at the TJ. Zona occludens (ZO)-1, ZO-2 and ZO-3 are the major TJ-plaque proteins, which bind to intracellular domain of occludin [4,5]. The interaction between occludin and ZO-1 plays a crucial role in maintaining the structure of TJ and epithelial barrier function [6]. On the other hand, E-cadherin (the transmembrane protein) and catenins (the AJ-plaque proteins) are the major proteins localized at the AJ [1]. Binding of $\beta$-catenin to E-cadherin plays an important role in the maintenance of the structure of AJs [7–9]. Additionally, a variety of intracellular signalling molecules have been localized at the TJ and AJ [1], which suggested the possible role of signalling pathways in the regulation of the structure and function of TJs and AJs. A significant body of evidence indicates that TJs and paracellular permeability are regulated by signalling molecules, such as intracellular calcium [10], cyclic AMP [11], rho GTPases [12,13], G-proteins [14,15] and protein kinases [16–26].

We have previously shown that oxidative stress increases the paracellular permeability in Caco-2 cell monolayers without affecting the cell viability [16,17]. This effect of oxidative stress was caused by glutathione oxidation and inhibition of protein tyrosine phosphatases [18]. Oxidative-stress-induced paracellular permeability was prevented by tyrosine kinase inhibitors, and was associated with tyrosine phosphorylation of a wide spectrum of proteins [16]. The specific role of protein tyrosine phosphorylation in the mechanism of the oxidative-stress-induced increase in paracellular permeability is not clear. However, evidence suggests that tyrosine phosphorylation of junctional proteins may play a role in regulation of cell–cell adhesion [20,27,28]. Therefore oxidative-stress-induced tyrosine phosphorylation may disrupt the protein complexes of TJs and AJs.

In the present study, we determined the effect of oxidative stress on occludin–ZO-1 and E-cadherin–$\beta$-catenin complexes, and analysed the tyrosine phosphorylation of these paracellular junctional proteins. This study shows that oxidative stress: (i) induces tyrosine phosphorylation of occludin, ZO-1, E-cadherin and $\beta$-catenin, (ii) dissociates the occludin–ZO-1 and E-cadherin–$\beta$-catenin complexes from the intercellular junctions by a tyrosine kinase-dependent mechanism, (iii) dissociates the occludin–ZO-1 complex, but not the E-cadherin–$\beta$-catenin complex, (iv) dissociates the occludin–ZO-1 and E-cadherin–$\beta$-catenin complexes from the cytoskeletal fraction by a tyrosine-kinase-dependent mechanism, and (v) induces tyrosine phosphorylation of cytoskeleton-associated ZO-1 and $\beta$-catenin.

## EXPERIMENTAL

### Chemicals

Cell culture media and related reagents were purchased from Gibco-BRL (Grand Island, NY, U.S.A.). Xanthine oxidase, xanthine, streptavidin agarose and protein-A Sepharose were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). All other chemicals were of analytical grade purchased either from Sigma Chemical Company or Fisher Scientific (Tustin, CA, U.S.A.).

Abbreviations used: AJ, adherens junction; HRP, horseradish peroxidase; TER, transepithelial electrical resistance; TJ, tight junction; XO+X, xanthine oxidase+xanthine; ZO, zonula occludens.

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**Table 1 Effect of genistein on XO + X-induced paracellular permeability**

The basal TER of monolayers varied from 400 to 500 $\Omega \cdot cm^2$. Values are means ± S.E.M. ($n = 4$). Results that are significantly ($P < 0.05$) different from values for control (*) or 3 h XO + X monolayers (†) are indicated.

| Property | 0 h | 1 h | 3 h | 3 h + genistein |
|---|---|---|---|---|
| Basal TER (%) | 103 ± 5 | 72 ± 4* | 30 ± 6* | 89 ± 7† |
| Mannitol flux (%/h/cm$^2$) | 0.21 ± 0.02 | 0.25 ± 0.02 | 0.85 ± 0.06* | 0.23 ± 0.02† |

### Antibodies

Mouse monoclonal anti-occludin, and rabbit polyclonal anti-occludin and anti-(ZO-1) antibodies were from Zymed Laboratories Inc. (South San Francisco, CA, U.S.A.). Mouse monoclonal anti-(E-cadherin) and anti-($\beta$-catenin), biotin-conjugated anti-phosphotyrosine and horseradish peroxidase (HRP)-conjugated anti-phosphotyrosine antibodies were from Transduction Laboratories (Lexington, KY, U.S.A.). FITC-conjugated anti-phosphotyrosine, Cy3-conjugated goat anti-rabbit IgG and FITC-conjugated anti-mouse IgG were from Sigma Chemical Co., and rabbit polyclonal anti-(E-cadherin) and anti-($\beta$-catenin) antibodies were from Chemicon International Inc. (Temecula, CA, U.S.A.).

### Cell culture

Caco-2 cells, purchased from the American Type Culture Collection (ATCC; Rockville, MD, U.S.A.), were maintained under standard cell culture conditions at 37 °C in medium containing 10% (v/v) foetal bovine serum. Cells were grown on polycarbonate membranes in Transwells (6.5 mm or 24 mm diameter; Costar, Cambridge, MA, U.S.A.), and experiments were performed on 12–14 days (6.5 mm diameter wells) or 20–22 days (24 mm diameter wells) after seeding.

### Treatment with oxidative stress

Confluent monolayers were bathed in Dulbecco's PBS containing 1.2 mM $CaCl_2$, 1 mM $MgCl_2$ and 0.6% (v/v) BSA. Oxidative stress was induced by administering (to both apical and basal compartments) a mixture of xanthine oxidase (20 m-units/ml) and xanthine (0.25 mM) (XO + X) with or without genistein (300 $\mu$M). Control cell monolayers were incubated in PBS without XO + X or genistein.

### Measurement of transepithelial electrical resistance (TER)

TER was measured according to the method of Hidalgo et al. [29] using a Millicell-ERS Electrical Resistance System (Millipore, Bedford, MA, U.S.A.). TER was calculated as $\Omega \cdot cm^2$ by multiplying the resistance by the surface area of the monolayer (0.33 cm$^2$). The resistance of the supporting membrane in Transwells (which is usually around 30 $\Omega \cdot cm^2$) was subtracted from all readings prior to calculations.

### Unidirectional flux of mannitol

Cell monolayers in Transwells were incubated under different experimental conditions in the presence of 0.2 $\mu$Ci/ml of D-[2-$^3$H]mannitol (15 Ci/mmol; ICN Biomedicals, Inc., Costa Mesa, CA, U.S.A.) in the basal well. At different times after oxidant administration, 100 $\mu$l each of apical and basal media were withdrawn and radioactivity was counted in a scintillation counter. The flux into the apical well was calculated as the percentage of total isotope administered into the basal well per hour per cm$^2$ of surface area.

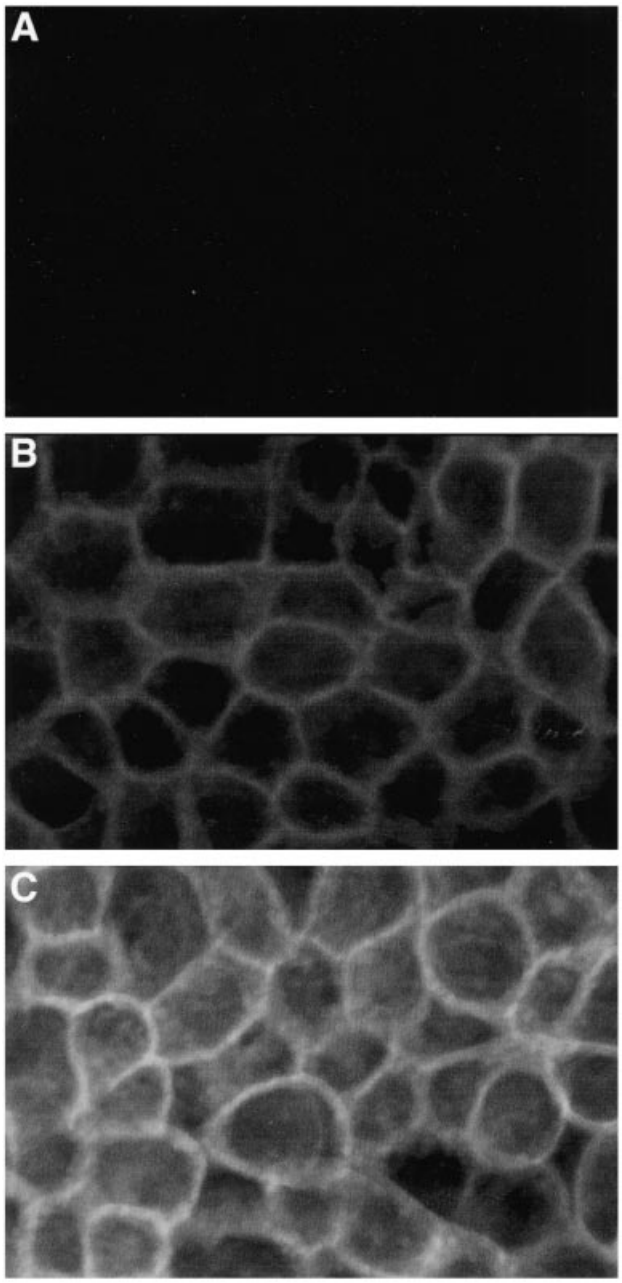


**Figure 1 Immunofluorescence localization of XO + X-induced protein tyrosine phosphorylation**

Caco-2 cell monolayers incubated with XO + X for 0 min (**A**), 15 min (**B**) or 30 min (**C**) were fixed and stained for phosphotyrosine using FITC-conjugated anti-phosphotyrosine antibody. Immunofluorescence was analysed by confocal laser scanning microscopy.

### Immunofluorescence microscopy

Under different experimental conditions, Caco-2 cell monolayers (6.5 mm diameter wells) were washed in PBS and fixed in acetone/methanol (1:1) at 0 °C for 5 min. Cell monolayers were

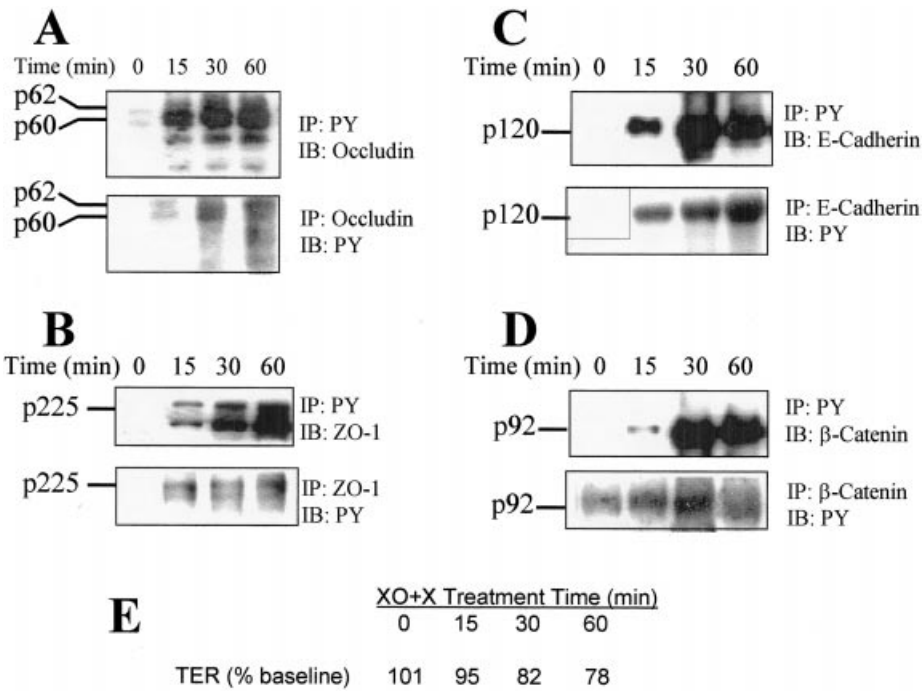


**Figure 2 XO + X-induced tyrosine phosphorylation of occludin, ZO-1, E-cadherin and β-catenin**

Caco-2 cell monolayers were treated with XO + X for various times, as indicated. Proteins were extracted under denaturing conditions and immunoprecipitated for phosphotyrosine using a biotin-conjugated anti-phosphotyrosine antibody. Immunoprecipitates were then analysed for occludin (**A**), ZO-1 (**B**), E-cadherin (**C**) and β-catenin (**D**) by immunoblot analysis using rabbit polyclonal anti-occludin and anti-(ZO-1), and mouse monoclonal anti-(E-cadherin) and anti-(β-catenin) antibodies. Alternatively, junctional proteins were immunoprecipitated followed by Western blot analysis for phosphotyrosine. Values in (**E**) represent the mean TER of the cell monolayers that were used. IB, immunoblotting; IP, immunoprecipitation; PY, phosphotyrosine.

blocked in TBST/BSA [20 mM Tris, pH 7.2, 150 mM NaCl and 1 % (v/v) BSA] and incubated with primary antibodies for 1 h, followed by incubation for 1 h with secondary antibodies (FITC-conjugated anti-mouse IgG and Cy3-conjugated anti-rabbit IgG antibodies). For staining of phosphotyrosine, cell monolayers were incubated directly with FITC-conjugated anti-phosphotyrosine antibody. Membranes were excised and mounted on a slide. The fluorescence was examined using a confocal laser scanning microscope (Bio-Rad MRC1024, Hercules, CA, U.S.A.), and series of images from 1 μm Y-sections were 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, U.S.A.). For vertical images, confocal Z-sections were analysed.

### Preparation of plasma membrane fractions

Caco-2 cell monolayers (24 mm diameter wells) 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 mM benzamidine and 1 mM PMSF). Cells were dispersed by homogenization in a Teflon/glass Dounce homogenizer with 50 strokes and lysed by sonication at 4 °C for two strokes (5 s each) with a 30 s interval. The cell lysate was centrifuged first at 3000 ***g*** for 10 min at 4 °C to sediment the cell debris. The supernatant was centrifuged further at 30000 ***g*** for 30 min at 4 °C. The pellet was suspended in 500 μl of lysis buffer F. After withdrawal of aliquots (2, 5 and 10 μl for high-density Triton-insoluble, low-density Triton-insoluble and Triton-soluble fractions respectively) for protein assay, the membrane fraction was mixed with an equal volume of 2 × Laemmli's sample buffer and heated at 100 °C for 5 min.

### Preparation of cytoskeletal fractions

Caco-2 cell monolayers (24 mm diameter wells) were washed twice with ice-cold PBS, and incubated for 5 min with lysis buffer CS (Tris buffer containing 1.0 % Triton X-100, 2 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml bestatin, 10 μg/ml pepstatin-A, 1 mM vanadate and 1 mM PMSF). Extracts were centrifuged first at 15600 ***g*** for 4 min at 4 °C to sediment the high-density actin cytoskeleton. The supernatant was further centrifuged at 100000 ***g*** for 4 h at 4 °C to sediment low-density membrane cytoskeleton [30]. The pellet was suspended in 200 μl of lysis buffer CS. After withdrawal of aliquots for protein assay, cytoskeletal fractions were mixed with an equal volume of 2 × Laemmli's sample buffer and heated at 100 °C for 5 min.

### Immunoprecipitation

After XO + X treatment, Caco-2 cell monolayers (24 mm diameter wells) were washed with ice-cold 20 mM Tris (pH 7.4) and the proteins were extracted in lysis buffer N (20 mM Tris, pH 7.4, containing 150 mM NaCl, 0.5 % Nonidet P40 and proteinase inhibitors as described above for lysis buffer F) at 4 °C for 30 min. Each cell monolayer was extracted in 0.75 ml of lysis buffer N, and extracts from two monolayers were pooled for each value for each experimental condition. Extracts were centrifuged at 3000 ***g*** for 10 min at 4 °C, and the supernatant,

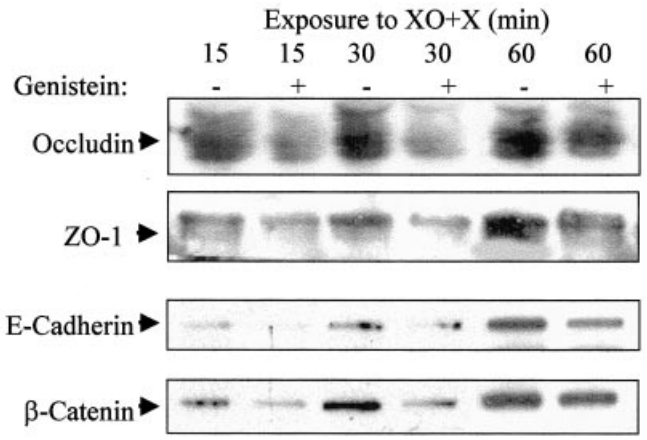


**Figure 3 Effect of genistein on XO + X-induced tyrosine phosphorylation of occludin, ZO-1, E-cadherin and β-catenin**

Caco-2 cell monolayers were treated with XO + X with or without genistein for various times. Proteins were extracted under denaturing conditions and immunoprecipitated for phosphotyrosine using biotin-conjugated anti-phosphotyrosine antibody. Immunoprecipitates were then analysed for occludin, ZO-1, E-cadherin and β-catenin by immunoblot analysis using rabbit polyclonal anti-occludin and anti-(ZO-1), and mouse monoclonal anti-(E-cadherin) and anti-(β-catenin) antibodies.

containing 1.0–1.5 mg protein/ml, was incubated with 2 μg of anti-occludin, anti-(ZO-1), anti-(E-cadherin) or anti-(β-catenin) antibodies at 4 °C for 15 h. Immune complexes were isolated by precipitation using protein-A Sepharose or protein-G Sepharose (for 1 h at 4 °C). Washed beads were suspended in 20 μl of Laemmli's sample buffer and heated at 100 °C for 5 min. Extracts were immunoblotted for occludin, claudin-1, ZO-1, E-cadherin or β-catenin, as described below, using specific primary and HRP-conjugated secondary antibodies.

For tyrosine-phosphorylation studies, proteins from whole cell or 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) under denaturing conditions (heating at 100 °C for 5 min). Phosphotyrosine was immunoprecipitated as described above using biotin-conjugated anti-phosphotyrosine antibody. Immune complexes were isolated by precipitation using streptavidin-agarose. Immunoprecipitates were immunoblotted for occludin, ZO-1, E-cadherin and β-catenin using antibodies, as described above. Alternatively, junctional proteins were immunoprecipitated using specific antibodies, followed by Western blot analysis for phosphotyrosine.

### Immunoblot analysis

Proteins were separated by SDS/PAGE (7.5 % gels) and transferred to nitrocellulose PVDF membranes. Membranes were blotted for occludin, ZO-1, E-cadherin and β-catenin using anti-occludin, anti-(ZO-1), anti-(E-cadherin) and anti-(β-catenin) antibodies in combination with HRP-conjugated anti-mouse IgG or HRP-conjugated anti-rabbit IgG antibodies. The blot was developed using the enhanced chemiluminescence method (ECL®, Amersham, Arlington Heights, IL, U.S.A.).

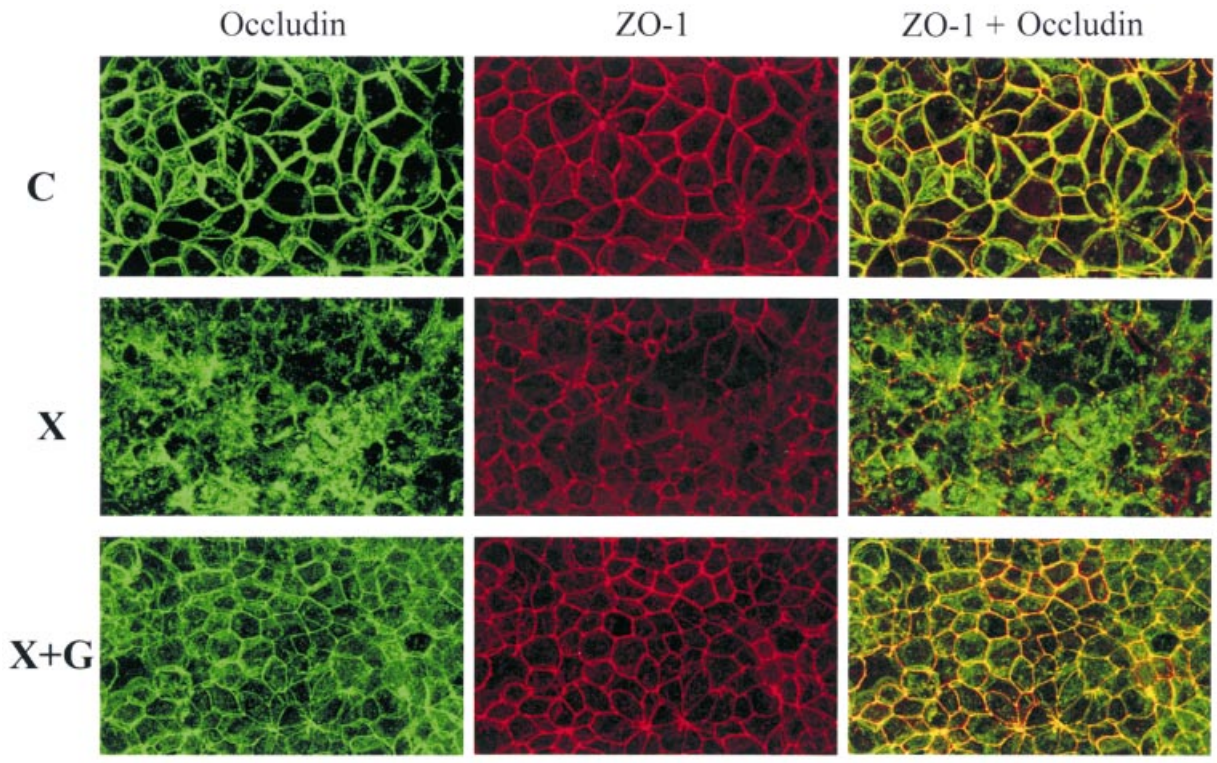


**Figure 4 Effect of XO + X on immunofluorescence localization of occludin and ZO-1: Y-sections**

Caco-2 cell monolayers were incubated for 3 h with PBS alone (C) or PBS containing XO + X without (X) or with (XG) genistein. Following incubation, cell monolayers were fixed and double-labelled for occludin (green) and ZO-1 (red) by immunofluorescence staining, as described in the Experimental section, using mouse monoclonal anti-occludin and rabbit polyclonal anti-(ZO-1) antibodies.

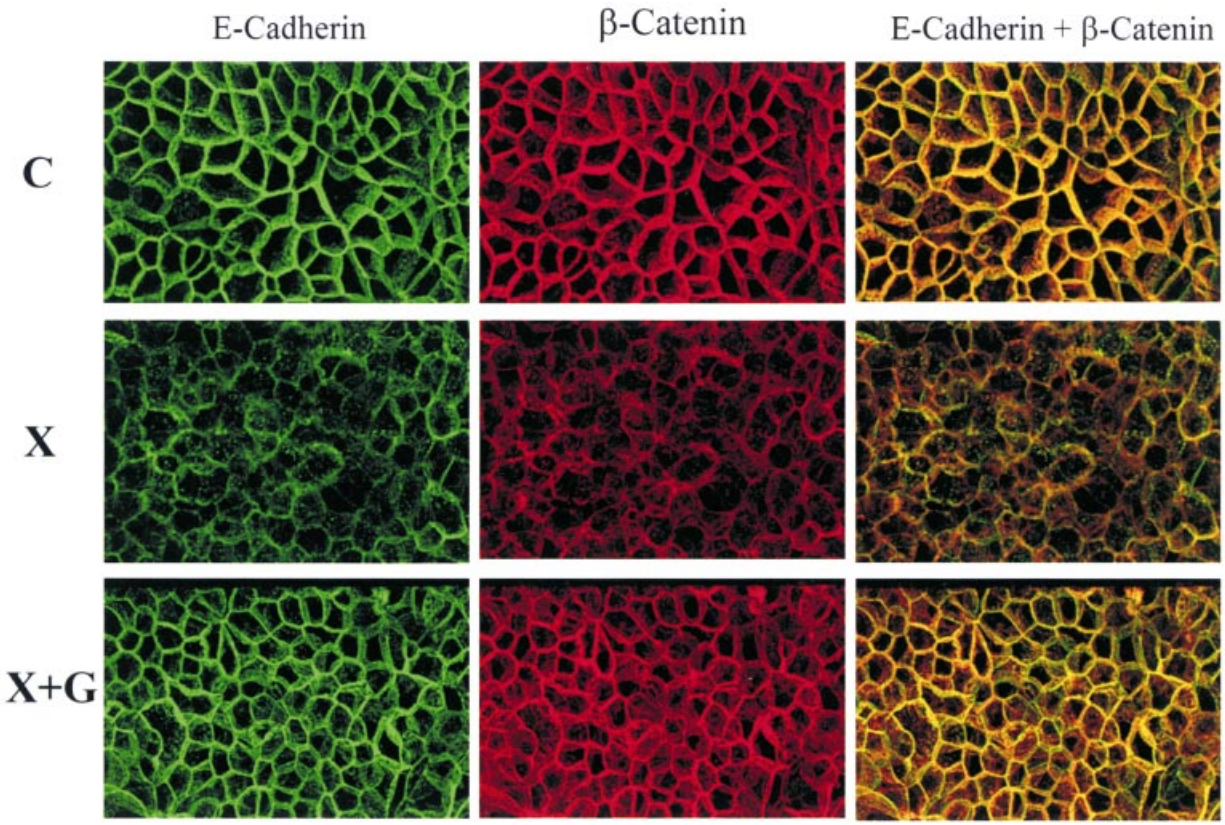


**Figure 5 Effect of XO + X on immunofluorescence localization of E-cadherin and β-catenin: Y-sections**

Caco-2 cell monolayers were incubated for 3 h with PBS alone (C) or PBS containing XO + X without (X) or with (X + G) genistein. Following incubation, cell monolayers were fixed and double-labelled for E-cadherin (green) and β-catenin (red) by immunofluorescence staining, as described in the Experimental section, using mouse monoclonal anti-(E-cadherin) and rabbit polyclonal anti-(β-catenin) antibodies.

### Statistics

Comparison between two groups was performed using Student's *t* test for grouped data in Table 1. The significance in all tests was derived at the 95% or greater confidence level.

## RESULTS

### Oxidative stress induces tyrosine phosphorylation of occludin, ZO-1, E-cadherin and β-catenin

Administration of XO + X into the buffer that incubated the Caco-2 cell monolayer resulted in a time-dependent decrease in TER and increase in mannitol permeability (Table 1). These changes in TER and mannitol permeability were prevented by co-administration of genistein, a tyrosine kinase inhibitor. Immunofluorescence staining of phosphotyrosine and confocal microscopy showed no detectable level of phosphotyrosine in the control cell monolayer (Figure 1), but there was a time-dependent increase in the fluorescence in XO + X-treated cell monolayers. The phosphotyrosine fluorescence was predominantly localized at the intercellular junctions. Immunoprecipitation of phosphotyrosine from denatured extracts of cell monolayers, followed by immunoblot analysis for specific junctional proteins, did not detect any tyrosine-phosphorylated occludin, ZO-1, E-cadherin or β-catenin in control cell monolayers (Figure 2). XO + X treatment resulted in a time-dependent increase in the levels of tyrosine-phosphorylated occludin, ZO-1, E-cadherin and β-catenin. Tyrosine phosphorylation of these proteins was detected as early as 15 min after XO + X administration and peak phosphorylation was achieved by 30 min. The presence of genistein, a tyrosine kinase inhibitor, reduced the XO + X-induced tyrosine phosphorylation of ZO-1, occludin, E-cadherin and β-catenin (Figure 3).

### Redistribution of occludin, ZO-1, E-cadherin and β-catenin by oxidative stress by a tyrosine-kinase-dependent mechanism

Immunofluorescence staining and confocal microscopy showed a co-localization of occludin and ZO-1 at the intercellular junctions of control cell monolayers (Figure 4). In XO + X-treated cell monolayers, fluorescence for occludin and ZO-1 was low at the intercellular junctions, while it was increased in the intracellular compartment. There was a partial disruption of the co-localization of occludin and ZO-1. The XO + X-induced redistribution of occludin and ZO-1 was decreased, or absent, in cell monolayers treated with XO + X in the presence of genistein. The fluorescence for E-cadherin and β-catenin was also co-localized at the intercellular junctions of control cell monolayers (Figure 5). In XO + X-treated cell monolayers, the fluorescence for E-cadherin and β-catenin at the intercellular junctions was less than that in control cell monolayers, however E-cadherin and β-catenin appear to be co-localized. Localization of E-cadherin and β-catenin at the intercellular junction in cell

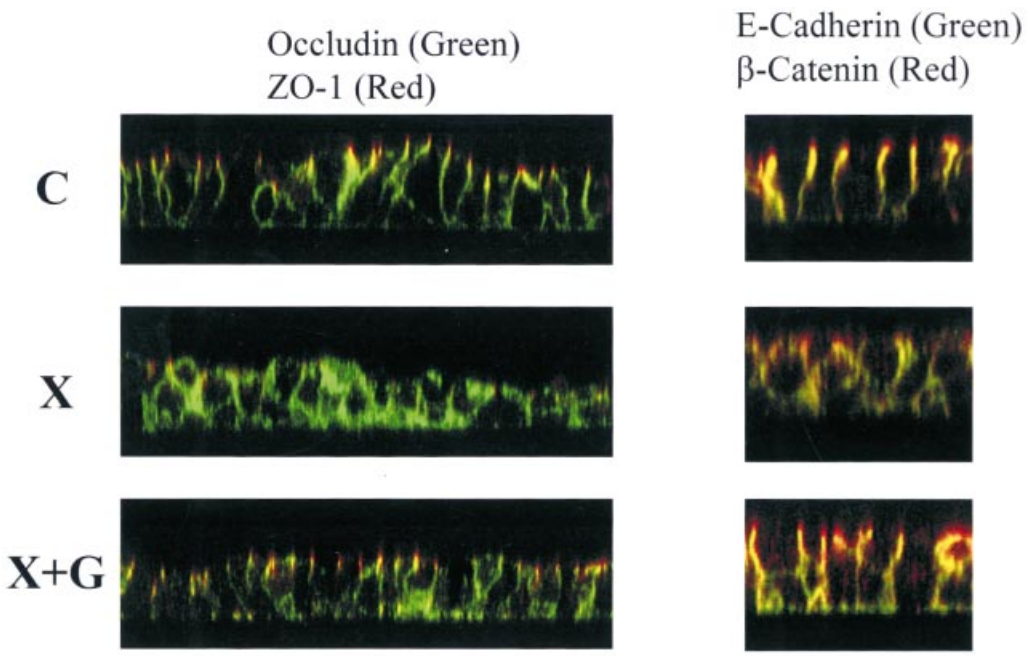


**Figure 6 Effect of XO + X on immunofluorescence localization of occludin–ZO-1 and E-cadherin–β-catenin complexes: Z-sections**

Caco-2 cell monolayers were incubated for 3 h with PBS alone (C) or PBS containing XO + X without (X) or with (X + G) genistein. Following incubation, cell monolayers were fixed and double-labelled for occludin (green) and ZO-1 (red) or for E-cadherin (green) and β-catenin (red) by immunofluorescence staining as described in the Experimental section. Fluorescence in Z-sections was examined by laser scanning confocal microscopy.

monolayers treated with XO + X and genistein was similar to that in control cell monolayers.

The Z-sections of fluorescence also showed a co-localization of ZO-1 and occludin at the apical end of intercellular junctions (Figure 6). The distribution of ZO-1 and occludin at the apical end of the junctions appeared to be disrupted and redistributed to the lateral and basal parts of the junctions in XO + X-treated cell monolayers. Similarly, the fluorescence for E-cadherin and β-catenin was also localized at the intercellular junctions, with a greater distribution at the apical half of cell junctions (Figure 6). The distribution of E-cadherin and β-catenin at the junction was disrupted in XO + X-treated cell monolayer; however, these two proteins continued to be co-localized. The junctional distribution in cell monolayers co-treated with XO + X and genistein was similar to that in control cell monolayers, except for a partial redistribution of occludin that remained unaffected by genistein.

### Oxidative stress disrupts the occludin–ZO-1 complex, but not the E-cadherin–β-catenin complex

To determine the effect of oxidative stress on binding of ZO-1 to occludin and β-catenin to E-cadherin, we analysed co-immunoprecipitation of these protein complexes. Immunoprecipitation of occludin from native extracts of control cell monolayers co-precipitated ZO-1 (Figure 7A). There was a decreased level of ZO-1 detected in anti-occludin immunoprecipitates prepared from XO + X-treated cell monolayers. However, ZO-1 precipitation with anti-occludin immunoprecipitates prepared from cell monolayers that were treated with XO + X and genistein was similar to that in control cell monolayers. Similarly, occludin was detected in immunoprecipitates of ZO-1 in control cell monolayers (Figure 7B). XO + X treatment decreased co-precipitation of occludin with ZO-1, which was prevented by genistein. Immunoprecipitation of E-cadherin co-precipitated β-catenin (Figure 7C), and immunoprecipitation of β-catenin co-precipitated E-cadherin (Figure 7D). However, co-precipitation of E-cadherin and β-catenin was no different in cell monolayers treated with XO + X in the presence or absence of genistein.

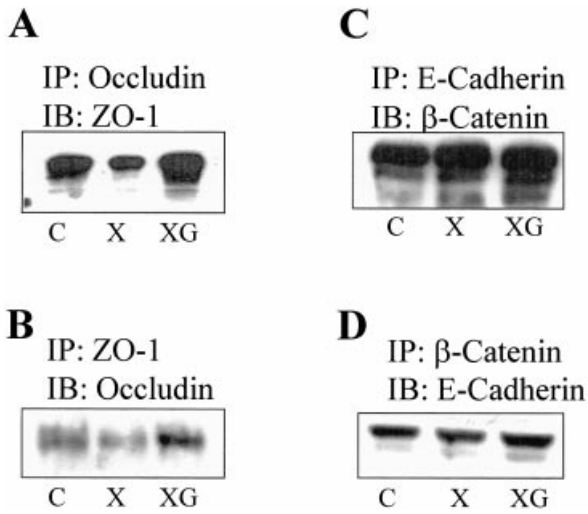


**Figure 7 Co-immunoprecipitation of occludin–ZO-1 and E-cadherin–β-catenin complexes**

Caco-2 cell monolayers were incubated without (C) or with XO + X in the absence (X) or presence (X + G) of genistein for 3 h. Proteins were extracted under native conditions and immunoprecipitated for occludin (**A**), ZO-1 (**B**), E-cadherin (**C**) or β-catenin (**D**) using specific antibodies and immunoblotted for ZO-1, occludin, β-catenin or E-cadherin respectively. IB, immunoblotting; IP, immunoprecipitation.

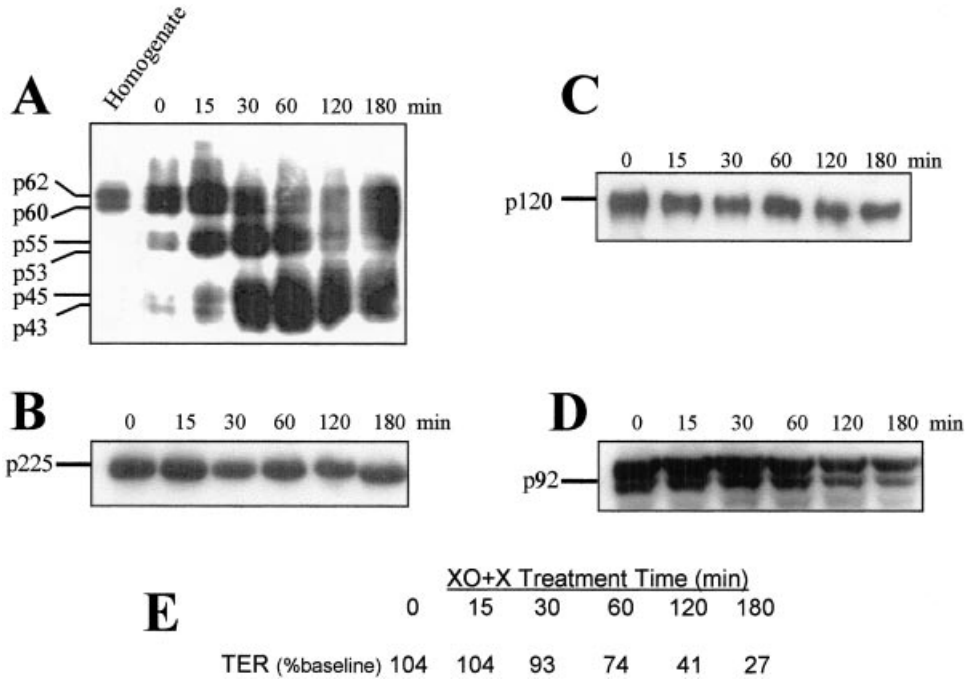


**Figure 8 Immunoblot analysis of occludin, ZO-1, E-cadherin and β-catenin in plasma membranes**

Caco-2 cell monolayers were treated with XO + X for various times, as indicated. Following treatment, plasma membranes were isolated and analysed by immunoblotting for occludin (**A**), ZO-1 (**B**), E-cadherin (**C**) and β-catenin (**D**), as described in the Experimental section, using mouse monoclonal anti-occludin, rabbit polyclonal anti-(ZO-1), mouse monoclonal anti-(E-cadherin) and rabbit polyclonal anti-(β-catenin) antibodies. The left-hand lane in (**A**) represents the occludin bands in the whole homogenate of untreated cell monolayers. Values in (**E**) represent the mean TER of cell monolayers used in this study.

### Oxidative stress induces proteolytic degradation of occludin and a partial reduction of E-cadherin and β-catenin in the plasma membrane fraction

Occludin and E-cadherin are transmembrane proteins, and ZO-1 and β-catenin bind to intracellular domains of occludin and E-cadherin respectively. The localization of occludin, ZO-1, E-cadherin and β-catenin in plasma membranes isolated from the control cell monolayers and the cell monolayers treated with XO + X was determined by immunoblot analysis. Occludin bands with molecular masses of 60 and 62 kDa were detected in plasma membrane fractions of control cell monolayer (Figure 8A). The intensity of this occludin band was reduced by XO + X treatment in a time-dependent manner. The XO + X-mediated decrease of the occludin band was associated with a corresponding appearance of new bands of occludin with molecular masses of 53 and 55 kDa, and 43 and 45 kDa. The intensity of low-molecular mass occludin bands appeared to be much greater than the high-molecular-mass occludin bands. This increased intensity of occludin bands may have been caused by a greater sensitivity of the antibody for binding to the dephosphorylated form of occludin (http://www.zymed.com; Zymed Laboratories, South San Francisco, CA, U.S.A.). A previous study has shown that occludin is highly phosphorylated at serine/threonine residues under basal conditions, and undergoes extensive dephosphorylation during the disruption of TJs [31]. ZO-1 was present in the plasma membrane fractions, and the intensity of this band was unaffected by XO + X treatment (Figure 8B). E-Cadherin (Figure 8C) and β-catenin (Figure 8D) were also present in the plasma membrane fractions of control cell monolayers. XO + X treatment induced only a slight decline in the plasma membrane-associated E-cadherin and β-catenin at 2 h or 3 h treatment.

Although genistein prevented XO + X-induced decrease in TER, it failed to prevent the XO + X-induced decrease in the molecular mass of occludin (Figure 9A). A slight decrease in plasma-membrane-associated E-cadherin and β-catenin by XO + X was also unaffected by genistein (Figure 9A). In the soluble fraction, the amount of ZO-1 was found to be greater in XO + X-treated cell monolayers when compared with that in control cell monolayers. Genistein prevented this increase in soluble ZO-1 by XO + X (Figure 9B). The amounts of E-cadherin and β-catenin in the soluble fraction of cells were not altered by XO + X or genistein. Similar to plasma membrane-associated occludin, soluble-fraction-associated occludin was also transformed to low-molecular-mass forms by XO + X, and this effect of XO + X was not prevented by genistein (Figure 9B).

Treatment of cell monolayers with XO + X in the presence of 1 mM 1,10-phenanthroline, a metalloproteinase inhibitor, prevented the XO + X-induced degradation of occludin in both plasma membrane and soluble fractions (Figure 9). However, 1,10-phenathroline strongly potentiated the effect of XO + X on TER (Figure 9C). It also decreased the plasma-membrane-associated ZO-1 and occludin, but had no effect on E-cadherin and β-catenin in plasma membrane or soluble fractions.

### Oxidative stress induces dissociation of cytoskeleton-bound occludin, ZO-1, E-cadherin and β-catenin by a tyrosine-kinase-dependent mechanism

ZO-1 is suggested to link transmembrane occludin with the actin cytoskeleton by binding to both occludin and F-actin [5]. Similarly, β-catenin links E-cadherin with F-actin [8]. To de-

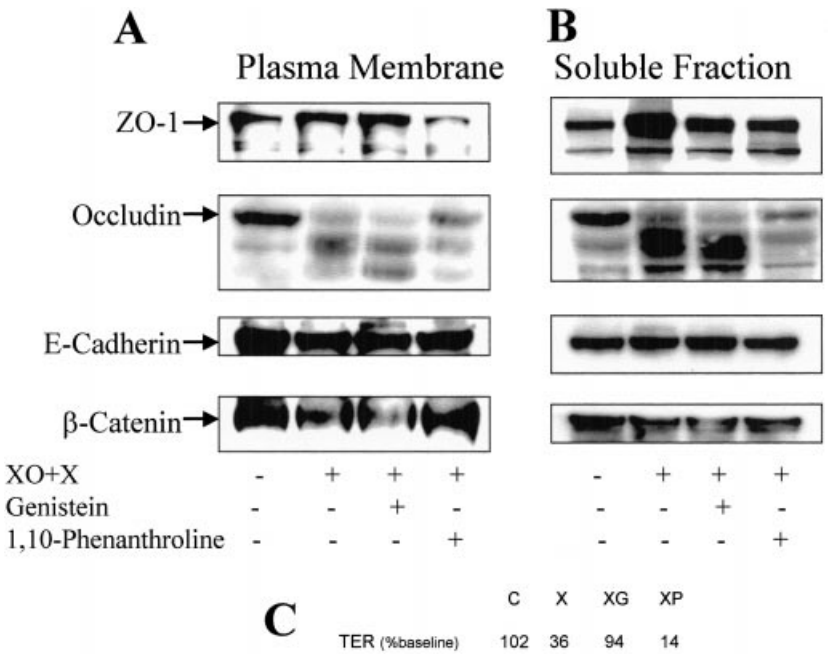


**Figure 9 Effect of genistein and 1,10-phenanthroline on XO + X-induced changes in plasma-membrane-associated ZO-1, occludin, E-cadherin and β-catenin**

Caco-2 cell monolayers were treated with XO + X in the presence or absence of genistein or 1,10-phenathroline. Control monolayers received no XO + X or genistein. Plasma membranes (**A**) and soluble fractions (**B**) were isolated after 3 h and examined for different proteins by immunoblot analysis. Results in (**C**) show mean TER values.

termine the effect of oxidative stress on cytoskeletal association of TJ and AJ proteins, we analysed the association of different junctional proteins with Triton-insoluble fractions in control and XO + X-treated cell monolayers. High-density Triton-insoluble fractions (cytoplasmic cytoskeleton) were rich in actin, whereas low-density Triton-insoluble fractions (membrane cytoskeleton) were rich in talin, as previously described [30]. High levels of occludin, ZO-1, E-cadherin and β-catenin were detected in the cytoplasmic cytoskeleton of control cell monolayers (Figure 10). XO + X treatment resulted in almost a complete depletion of occludin, and a partial decrease of ZO-1 in the cytoplasmic cytoskeletal fraction. This effect of XO + X was completely prevented by the presence of genistein. XO + X treatment also partially decreased cytoskeleton-bound E-cadherin and β-catenin, which was prevented by the presence of genistein.

Occludin, ZO-1, E-cadherin and β-catenin were also detected in the membrane cytoskeleton fraction (Figure 10). ZO-1, E-cadherin and β-catenin in the membrane cytoskeleton fraction were not affected by XO + X, but were slightly decreased by genistein. Occludin in both the membrane cytoskeleton and Triton-soluble fractions was degraded to low-molecular-mass forms by XO + X treatment (Figure 10). Genistein did not prevent XO + X-induced degradation of occludin in either the membrane cytoskeleton or Triton-soluble fraction. The amount of ZO-1 in the Triton-soluble fraction was greater in XO + X-treated cell monolayers than in control cell monolayers or cell monolayers treated with XO + X in the presence of genistein. E-Cadherin and β-catenin in Triton-soluble fractions were not affected by XO + X or genistein.

Immunoprecipitation of phosphotyrosine in Triton-soluble and insoluble fractions, followed by Western blot analysis for junctional proteins, shows that XO + X treatment induced tyrosine phosphorylation of ZO-1 and β-catenin associated with cytoplasmic actin cytoskeleton (Figure 11). Actin-associated E-cadherin was only slightly phosphorylated, while actin-associated occludin was phosphorylated in the absence of XO + X, which was decreased by treatment with XO + X. This decrease in tyrosine-phosphorylated occludin from the actin cytoskeleton may have been caused by the release of occludin from the cytoskeleton by oxidative stress. XO + X induced a time-dependent increase in tyrosine phosphorylation of occludin associated with membrane cytoskeleton, but only a transient increase in tyrosine phosphorylation of ZO-1, E-cadherin and β-catenin. In the Triton-soluble fraction, XO + X increased tyrosine phosphorylation of all proteins, predominantly that of E-cadherin.

## DISCUSSION

A significant body of evidence suggests that intracellular signalling pathways have an important role in regulation of the structure and function of TJs and AJs. Our studies [16,17] have demonstrated that oxidative stress increases paracellular permeability of Caco-2 cell monolayer by a mechanism involving protein tyrosine phosphorylation. In the present study, we show that oxidative-stress-induced increase in paracellular permeability is associated with tyrosine phosphorylation of occludin, ZO-1, E-cadherin and β-catenin. Moreover, oxidative stress disrupts occludin–ZO-1 and E-cadherin–β-catenin complexes from the intercellular junctions and their association with cytoskeleton by a tyrosine-kinase-dependent mechanism. Dissociation from the cytoskeleton and redistribution of occludin–ZO-1 and E-cadherin–β-catenin complexes indicate that oxidative stress disrupts TJs and AJs of the Caco-2 cell monolayer by protein tyrosine phosphorylation. Tyrosine phosphorylation of occludin, ZO-1, E-cadherin and β-catenin suggests that tyrosine phosphorylation of these proteins may have a role in the mechanism of oxidative-stress-induced dissociation of occludin–

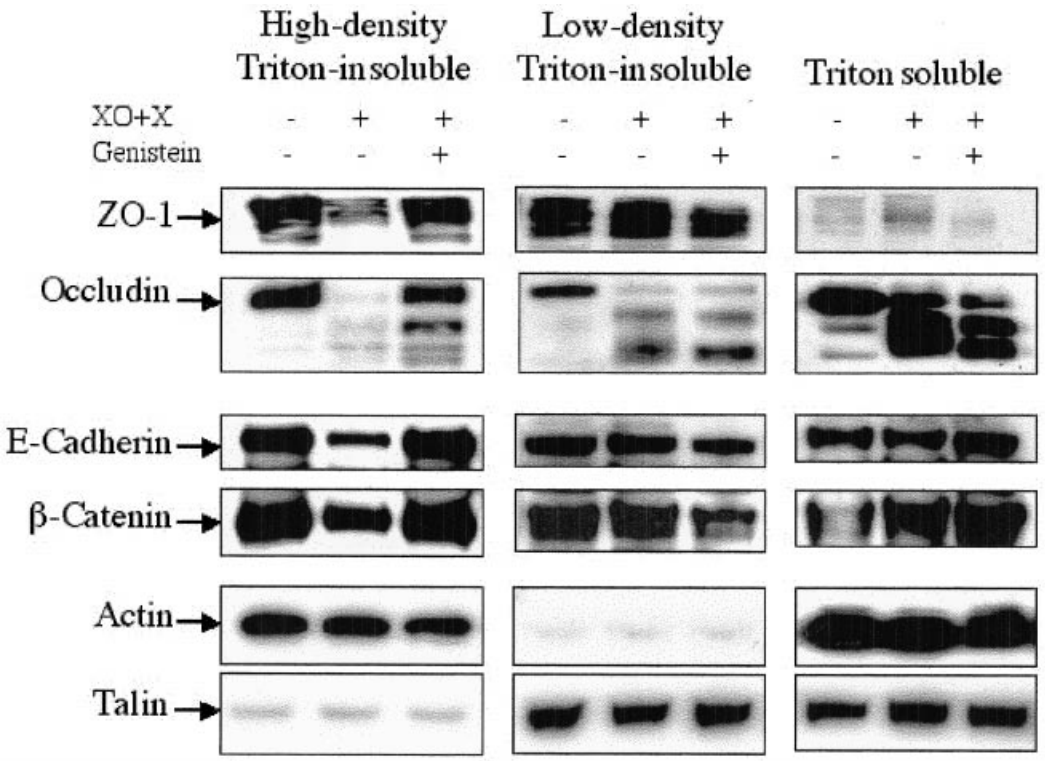


**Figure 10 Effect of XO + X and genistein on the association of ZO-1, occludin, E-cadherin and β-catenin in high density Triton-insoluble, low density Triton-insoluble and Triton-soluble fractions**

Caco-2 cell monolayers were treated for 3 h with XO + X in the presence or absence of genistein. Control monolayers received no XO + X or genistein. High-density Triton-insoluble (cytoplasmic cytoskeleton), low-density Triton-insoluble (membrane cytoskeleton) and Triton-soluble fractions were isolated and examined for different proteins by immunoblot analysis. Actin and talin were analysed as markers for cytoplasmic cytoskeleton and membrane cytoskeleton fractions respectively.

ZO-1 and E-cadherin–β-catenin complexes from the cytoskeleton and disruption of TJs and AJs.

Our previous study demonstrated that oxidative stress induces a rapid increase in tyrosine phosphorylation of clusters of proteins in Caco-2 cell monolayers [16]. However, the identity of any of these proteins is not known. Immunofluorescence localization of phosphotyrosine in the present study confirms an oxidative-stress-induced increase in protein tyrosine phosphorylation. The location of phosphotyrosine at the intercellular junctions suggested that oxidative stress phosphorylates the proteins associated with TJs and AJs. The present study demonstrates that oxidative stress induces a rapid increase in tyrosine phosphorylation of occludin, ZO-1, E-cadherin and β-catenin. Tyrosine phosphorylation of E-cadherin and β-catenin has been previously shown to prevent the binding of β-catenin to E-cadherin and cadherin-based cell–cell adhesion [28,32,33]. Tyrosine phosphorylation of occludin and ZO-1 suggests that tyrosine phosphorylation may mediate disruption and redistribution of the occludin–ZO-1 complex from TJs, leading to an increase in paracellular permeability. Results also show that genistein, a tyrosine kinase inhibitor, reduces the oxidative-stress-induced tyrosine phosphorylation of junctional proteins.

Immunofluorescence localization of occludin and ZO-1 at the intercellular junctions indicates the presence of well defined TJs in Caco-2 cell monolayers, which contribute to a TER of 400–500 $\Omega \cdot cm^2$. Occludin is the first transmembrane protein localized at the TJ [1]. At the intracellular C-terminal domain, occludin binds to ZO-1, ZO-2 and ZO-3, and the binding of ZO-1 to occludin is well characterized as being required for the maintenance of the integrity of TJs [6]. In the present study, we show that oxidative stress causes a reduction in ZO-1 stain (by immunofluorescence) at the intercellular junctions and a redistribution of occludin from the intercellular junctions. This observation indicates that the interaction between ZO-1 and occludin is disrupted by oxidative stress. Disruption of the occludin–ZO-1 complex was further confirmed by a loss of co-immunoprecipitation of ZO-1 and occludin in oxidative-stress-treated cell monolayer. The prevention of oxidative-stress-induced redistribution and the loss of interaction between occludin and ZO-1 by genistein indicates the role of tyrosine kinase activity in disruption of occludin–ZO-1 complex. Immunofluorescence staining also showed a dissociation and redistribution of the AJ-specific proteins, E-cadherin and β-catenin, from the intercellular junctions. Unlike the occludin–ZO-1 complex, the E-cadherin–β-catenin complex was maintained and redistributed without dissociation. This was confirmed by the lack of effect of oxidative stress on co-immunoprecipitation of E-cadherin and β-catenin.

ZO-1 establishes a link between transmembrane occludin and cytoskeleton [5]. Interaction with the cytoskeleton anchors occludin at the TJ, and this interaction is crucial for the maintenance of the structure and function of TJs. Disruption of cytoskeleton by cytochalasin-D disrupts TJs and increases paracellular permeability [34]. In the present study, we show that occludin and ZO-1 are associated with the isolated cytoskeletal fraction. Oxidative stress almost completely releases occludin

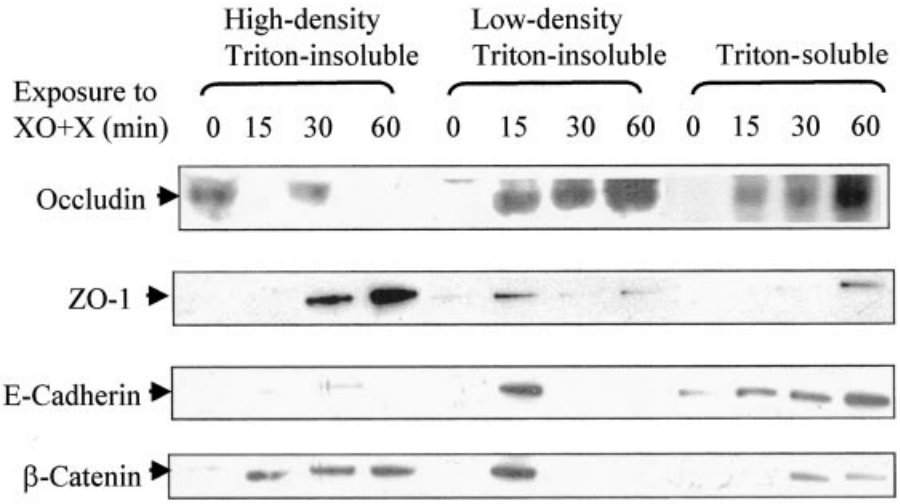


**Figure 11 XO + X-induced tyrosine phosphorylation of occludin, ZO-1, E-cadherin and β-catenin in Triton-insoluble and Triton-soluble fractions**

Caco-2 cell monolayers were treated with XO + X for various times, as indicated. High-density Triton-insoluble, low-density Triton-insoluble and Triton-soluble fractions were prepared. Proteins in different fractions were extracted under denaturing conditions and immunoprecipitated for phosphotyrosine using a biotin-conjugated anti-phosphotyrosine antibody. Immunoprecipitates were then analysed for occludin, ZO-1, E-cadherin and β-catenin by immunoblot analysis.

from the cytoskeleton and partially dissociates cytoskeletal ZO-1. Similarly, cytoskeleton-associated E-cadherin and β-catenin were partially released by oxidative stress. However, oxidative stress failed to alter plasma-membrane-associated ZO-1, whereas E-cadherin and β-catenin in plasma membrane were only slightly decreased. Although it undergoes proteolytic degradation, the intact occludin and its degradation products continue to be associated with the plasma membrane fraction of oxidative-stress-induced cell monolayers. These observations indicate that oxidative-stress-induced disruption of TJs and AJs is mainly caused by the dissociation of interaction between cytoskeleton and the occludin–ZO-1 and E-cadherin–β-catenin complexes, rather than the release of these proteins from the plasma membrane. A complete prevention of oxidative-stress-induced release of cytoskeleton-associated occludin–ZO-1 and E-cadherin–β-catenin complexes by genistein demonstrates that tyrosine phosphorylation plays a role in disrupting the interaction of occludin–ZO-1 and E-cadherin–β-catenin complexes with the actin cytoskeleton. Oxidative stress induced tyrosine phosphorylation of ZO-1 and β-catenin that are associated with actin cytoskeleton, whereas E-cadherin was only slightly phosphorylated. Occludin in actin cytoskeleton was phosphorylated under basal conditions, whereas its phosphorylation was decreased by oxidative stress. Decrease in tyrosine phosphorylated occludin may have been caused by the release of occludin from the actin cytoskeleton. In the membrane cytoskeleton, oxidative stress induced tyrosine phosphorylation of occludin and transient phosphorylation of ZO-1, E-cadherin and β-catenin. These results suggest that tyrosine phosphorylation of ZO-1 and β-catenin may play a crucial role in the oxidative-stress-induced disruption of occludin–ZO-1 and E-cadherin–β-catenin complexes.

On the other hand, genistein failed to prevent oxidative-stress-induced degradation of occludin or the minor decrease in E-cadherin and β-catenin in the plasma membrane fraction. Interestingly, the decrease in occludin in the plasma membrane fraction was accompanied by a time-dependent appearance of low-molecular-mass occludin bands (43 and 45 kDa, and 53 and 55 kDa). Phenylarsine oxide, an inhibitor of protein tyrosine phosphatases, disrupted TJs of endothelial cell monolayers by proteolytic degradation and redistribution of occludin, but not ZO-1, E-cadherin or β-catenin. Occludin redistribution and TJ permeability by phenylarsine oxide was partially prevented by a metalloproteinase inhibitor [35]. However, in the present study we show that oxidative-stress-induced occludin degradation plays no role in disruption of TJs. The metalloproteinase inhibitor 1,10-phenanthroline prevented the occludin degradation by oxidative stress, but it potentiated the oxidative-stress-induced decrease in TER.

In summary, the present study shows that oxidative stress induces tyrosine phosphorylation of occludin–ZO-1 and E-cadherin–β-catenin complexes, and results in redistribution of these protein complexes in Caco-2 cell monolayers. Tyrosine-kinase-dependent dissociation of occludin–ZO-1 and E-cadherin–β-catenin complexes from the cytoskeleton appears to be the main mechanism involved in oxidative-stress-induced disruption of TJs and AJs, and increase in paracellular permeability.

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