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Iron and copper alter tight junction permeability in human intestinal Caco-2 cells by distinct mechanisms

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Abstract

Human intestinal Caco-2 cells differentiated for 15–17 days on transparent filter inserts were treated for up to 3 h with 50 and 100 μM $CuCl_2$ or $FeSO_4$ in the AP compartment at pH 6.0. Trans-epithelial electrical resistance (TEER) showed a progressive decrease during the course of the experiment that was slower in cells treated with 50 μM $CuCl_2$ than in those treated with 100 μM $CuCl_2$. Both 50 and 100 μM $FeSO_4$ produced a similar decrease in TEER over time, tailing off after 120 min. F-actin localization by fluorescent phalloidin binding in control cells and in cells treated for 3 h with 50 μM $CuCl_2$ or $FeSO_4$ highlighted striking differences in the two treatments. Cu(II) led to an overall reduction in F-actin staining with extensive depolymerization in areas of the monolayer, in the absence of cellular loss. Conversely, Fe(II) treatment produced disorganization of F-actin and decreased staining of the perijunctional actin filaments. No changes in the localization and intensity of staining of the junctional proteins ZO1, occludin and E-cadherin were observed after treatment with 100 μM $FeSO_4$ in analogy with previous observations in Cu(II)-treated cells. The data presented suggest that different mechanisms are responsible for the changes to tight junction permeability produced by the two metals.

Keywords: F-actin; Trans-epithelial electrical resistance; Ferrous sulphate; ZO1; Occludin; E-cadherin

1. Introduction

Iron and copper are essential nutrients, yet they can become toxic above certain doses. They are extensively used as dietary integrators by large sections of the population in Western countries, often without medical supervision. Their possible toxicity at the level of the gastrointestinal tract is therefore of interest, especially since the intestinal mucosa represents a potential site of entry of toxic substances into the blood circulation.

The human intestinal Caco-2 cell line, grown and differentiated on permeable filter supports, represents a useful model for the study of the effects of toxic substances, including heavy metals (Baker et al., 1995; Rossi et al., 1996; De Angelis et al., 1998; Ma et al., 1999). In particular, alterations in permeability produced by changes at the level of the tight junctions represent a sensitive indicator of sublethal toxic effects at the level of the intestinal mucosa. We have previously reported that copper can induce a concentration- and time-dependent increase in tight junction permeability in Caco-2 cells, that was reversible after treatment with up to 100 μM $CuCl_2$ for 3 h and was accompanied by depolymerization of the F-actin cytoskeleton (Ferruzza et al., 1999a,b).

Since iron is given in relatively high doses as a dietary supplement in certain physiological conditions, including pregnancy and old-age, it is particularly important to establish the effects of iron on the tight junctions of intestinal cells. The aim of this work was therefore to compare the effects of the two heavy metals, iron and copper, on the tight junction permeability, on the F-actin cytoskeleton and on junctional proteins in differentiated human intestinal Caco-2 cells.

2. Materials and methods

The human intestinal Caco-2 cell line was obtained from Professor Alain Zweibaum (INSERM, Villejuif, Paris, France). Caco-2 cells were grown and maintained as previously described (Ferruzza et al., 1999b) in Dul-

Abbreviations: AP, apical; BL, basolateral; BSS, balanced salt solution; FITC, fluorescein isothiocyanate; GSH, reduced glutathione; MES, morpholinoethane sulfonic acid; PBS, phosphate buffered saline; ROS, reactive oxygen species; TEER, trans-epithelial electrical resistance

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becco's modified minimum essential medium containing 25 mM glucose, 3.7 g/l $NaHCO_3$ and supplemented with 4 mM L-glutamine, 1% non-essential amino acids, 1×10^5 U/l penicillin, 100 mg/l streptomycin and 10% heat-inactivated fetal calf serum (complete culture medium). The cells were seeded on transparent filters (P.E.T. track-etched membrane, 25 mm diameter, 4.71 cm^2 area, 0.4 μm pore diameter; Becton Dickinson Labware Europe, Meylan Cedex, France) at a density of 4×10^5 cells/cm^2 and were left to differentiate for 15–17 days after confluence; the medium was regularly changed three times a week.

Cells were treated from the apical (AP) side with balanced salt solution (BSS; 137 mM NaCl, 5.36 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose) containing 20 mM morpholinoethane sulfonic acid (MES) pH 6.0 with or without the addition of 50 and 100 μM $CuCl_2$ or $FeSO_4$. The basolateral (BL) compartment contained BSS additioned with 20 mM HEPES at pH 7.4. These pH conditions were chosen to reproduce the pH gradient existing in vivo across the mucosa of the small intestine. During treatment, the permeability of the cell monolayer was monitored by measuring the trans-epithelial electrical resistance (TEER) of the cell monolayers at 37 °C, using a commercial apparatus (Millicell ERS; Millipore Co., Bedford, MA, USA) as previously described (Ferruzza et al., 1999b). TEER was expressed as Ω cm^2 after subtracting from the reading the resistance of the supporting filter and multiplying it by the surface area of the monolayer. At the end of the experiment, cells were washed with phosphate buffered saline with 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS^+) and fixed for morphological studies.

For-F-actin localization cells were fixed in 2% paraformaldehyde in PBS^+ containing 2% sucrose for 30 min, washed with PBS^+, blocked with 5% non-fat dry milk in PBS^+ and incubated for 30 min with 0.33 μg/ml fluorescein isothiocyanate (FITC)–phalloidin (0.25 μM) in PBS^+ containing 0.2% BSA; double-labeling of the nuclei was performed by incubating the filters for 30 min at 37 °C in 20 μg/ml RNAse A (from bovine pancreas, Boehringer Mannheim Italia, Monza, Italy, previously boiled for 10 min to denature DNAse), and staining with 0.001% propidium iodide (Sigma-Aldrich, Milano, Italy) in PBS^+ for 10 s.

For immunofluorescent localization of junctional proteins, cells were fixed in methanol at −20 °C for 2 min, washed with PBS^+, blocked with 5% non-fat dry milk in PBS^+ and treated with primary antibodies and secondary FITC-conjugated antibodies (Cappel-Organon Tecknika Co., Durham, NC, USA) according to conventional techniques. The primary antibodies used were rabbit polyclonals anti-ZO1 and anti-occludin and mouse monoclonal anti-E-cadherin (Zymed Laboratories Inc., S. Francisco, CA, USA). Filters were mounted cells-side up in Vectashield medium (Vector Laboratories, Burlingame, CA, USA). The cells were viewed with a fluorescent microscope (Axioscope 2, C. Zeiss; Jena, Germany).

3. Results

The functional effects of copper (II) and of iron (II) on the permeability of tight junctions in differentiated Caco-2 cells were monitored by measuring TEER values during treatment with 50 and 100 μM $CuCl_2$ or $FeSO_4$ from the AP side at pH 6.0 for 3 h. As shown in Fig. 1, Cu(II) ions decreased TEER values in a dose-dependent fashion as evidenced by the faster and larger decrease in TEER values observed after treatment with 100 μM than with 50 μM $CuCl_2$. Conversely, Fe(II) treatment produced a similar decrease in TEER values at both concentrations tested that reached a plateau value after 120 min. At the end of the treatment, the cells were fixed for morphological studies. Staining of the actin cytoskeleton with FITC-labeled phalloidin highlighted differences in the amount and in the distribution of F-actin in control and in Fe(II) and Cu(II) treated cells (Plate 1). Control cells exhibited a regular distribution of F-actin with strong staining along the cell periphery, corresponding to the perijunctional actin ring, and in the cytoplasm (A). After treatment with 50 μM $FeSO_4$, the F-actin signal in the cytoplasm appeared more diffuse and a strong reduction in the perijunctional actin ring was observed (C). Conversely, treatment with 50 μM $CuCl_2$ resulted in an overall reduction in the cytoplasmic F-actin signal with large areas of the cell monolayer exhibiting almost complete loss of staining (E). The uniform and regular staining of the nuclei in the same microscopic fields after both Fe(II) (D) or Cu(II) treatment (F) was indistinguishable from that in control cells (B), ruling out the possibility that the reduction in F-actin staining was a consequence of cell loss.

The localization of junctional proteins was investigated in control Caco-2 cells and in cells treated for 3 h with 100 μM $FeSO_4$ (Plate 2). The tight junctional proteins ZO1 (A, B) and occludin (C, D), and the protein of the adherens junction E-cadherin (E, F) all showed a regular and continuous staining around the cell periphery, with no detectable differences between control (A, C, E) and Fe(II)-treated cells (B, D, F). The modifications in F-actin observed in cells treated with 100 μM $FeSO_4$ were very similar to those observed after treatment with 50 μM $FeSO_4$.

4. Discussion

Iron and copper ions are able to alter the barrier function of the intestinal mucosa as shown by the

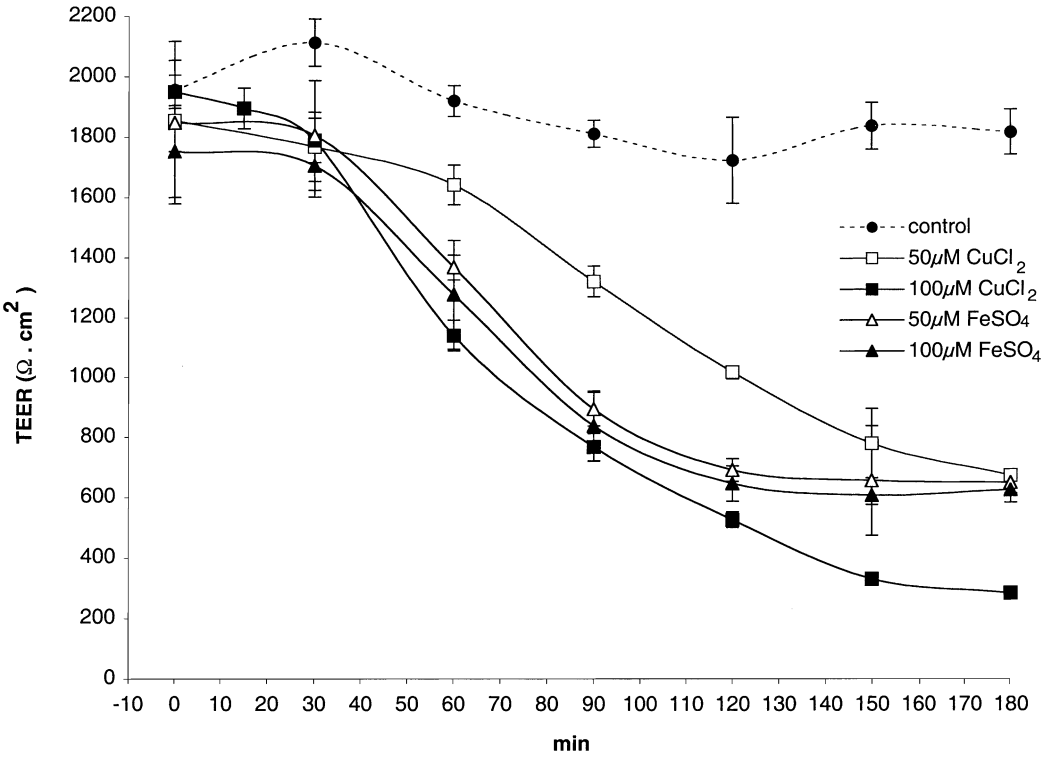

Fig. 1. Time course of TEER changes recorded in control Caco-2 cells and in cells treated from the AP side with 50 and 100 μM $CuCl_2$ or $FeSO_4$ at pH 6.0. Data are the mean±S.D. of an experiment performed in triplicate.

increase in tight junction permeability observed after treatment of Caco-2 cells with $FeSO_4$ and $CuCl_2$ for 3 h. In our experiments, treatment with 50 μM $FeSO_4$ led to a faster decrease of TEER values than that obtained with the same concentration of $CuCl_2$. Despite these differences, however, the two metals exhibited comparable functional effects on tight junction permeability in terms of dose and time dependence.

We have previously characterized the effects of copper on tight junction permeability. The increase in permeability induced by copper was shown to be directly related to the cellular uptake of the metal and was reversible (up to 100 μM $CuCl_2$ for 3 h) following de novo RNA transcription and protein translation (Ferruzza et al., 1999b). Conversely, preliminary data indicate that treatment with $FeSO_4$ between 25 and 100 μM for 3 h does not lead to recovery of the TEER values after 24 h in complete culture medium (data not shown). The lack of recovery of TEER values after treatment with $FeSO_4$ was accompanied by extensive cell detachment from the filter. This is an indication that, whatever mechanism initiated the changes to tight junction permeability, more extensive intracellular damage occurred during the recovery period, leading to cell death by either apoptosis or necrosis. Further work is required to elucidate the mechanisms involved.

The effects of Fe(II) treatment on tight junction permeability in Caco-2 cells have, to the best of our knowledge, only been reported in Courtois et al. (2000), showing that treatment with 50–400 μM Fe(II)-ascorbate for 24 h resulted in lipid peroxidation, as monitored by an increase in malondialdehyde, but did not alter TEER values. These results are in contrast with our observations that 50–100 μM Fe(II)-sulfate (Fig. 1) as well as Fe(II)-ascorbate (data not shown) produce a similar decrease in TEER values after 3 h of treatment. This discrepancy is difficult to explain and may arise from differences in the experimental procedures such as the preparation of the Fe(II)–ascorbate complex (that is extremely unstable), the pH in the AP compartment (6.0 vs 7.4) or the medium (saline solution vs culture medium) used.

Both Fe(II) and Cu(II) are potent generators of reactive oxygen species (ROS) in aqueous solution. Several reports in the literature have shown that free radical damage in Caco-2 cells is accompanied by an increase in tight junction permeability (Baker et al., 1995; Manna et al., 1997; Rao et al., 1997, 2000; Banan et al., 2000a, 2001) However, not all free radical species are effective in altering tight junction permeability, as shown in Rao et al. (2000). The hyperpermeability of tight junctions induced by H_2O_2 or by nitric oxide under acidic conditions has been associated with a decrease in intracellular reduced glutathione (GSH) (Unno et al., 1997; Rao et al., 2000), this in turn leading to changes in tyrosine kinase and/or protein tyrosine phosphatase activities that modify the phosphorylated state of junctional proteins. Further work is required to determine whether

Fe(II) treatment affects tight junction permeability by a similar mechanism. In the case of copper, neither intracellular (Trolox, DMSO) nor extracellular (mannitol) antioxidants were able to reduce the decrease in TEER observed after treatment with $CuCl_2$ (Ferruzza et al., 1999a), thus ruling out, at least under the experimental conditions used, an involvement of oxidative damage in the copper effect.

Another striking difference between Fe(II) and Cu(II) was observed at the structural level in the localization of F-actin. While Cu(II) induced depolymerization of F-actin especially in the terminal web (Plate 1; Ferruzza et al., 1999b), treatment with Fe(II) led to an apparent disorganization of the F-actin cytoskeleton with disappearance of the perijunctional ring but without obvious signs of depolymerization. Despite such changes, the localization of junctional proteins ZO1, occludin and E-cadherin, appeared unaffected by treatment with either metal (Plate 2; and Ferruzza et al., 1999b).

The effect of copper on F-actin depolymerization does not appear to be a consequence of oxidative damage (as discussed above) and the kinetics and characteristics of recovery indicate that de novo synthesis of cellular components is required, suggesting a direct effect of copper on F-actin itself or on some factor involved in actin filaments maintenance and assembly. Conversely, the different appearance of the F-actin cytoskeleton on Fe(II) treatment points towards disorganization and delocalization of the perijunctional actin filaments, rather than to extensive depolymerization. Carbonylation of F-actin as well as of α-tubulin has been reported in Caco-2 cells rendered hyperpermeable by treatment with reactive oxygen metabolites (Banan et al., 2000a,b,

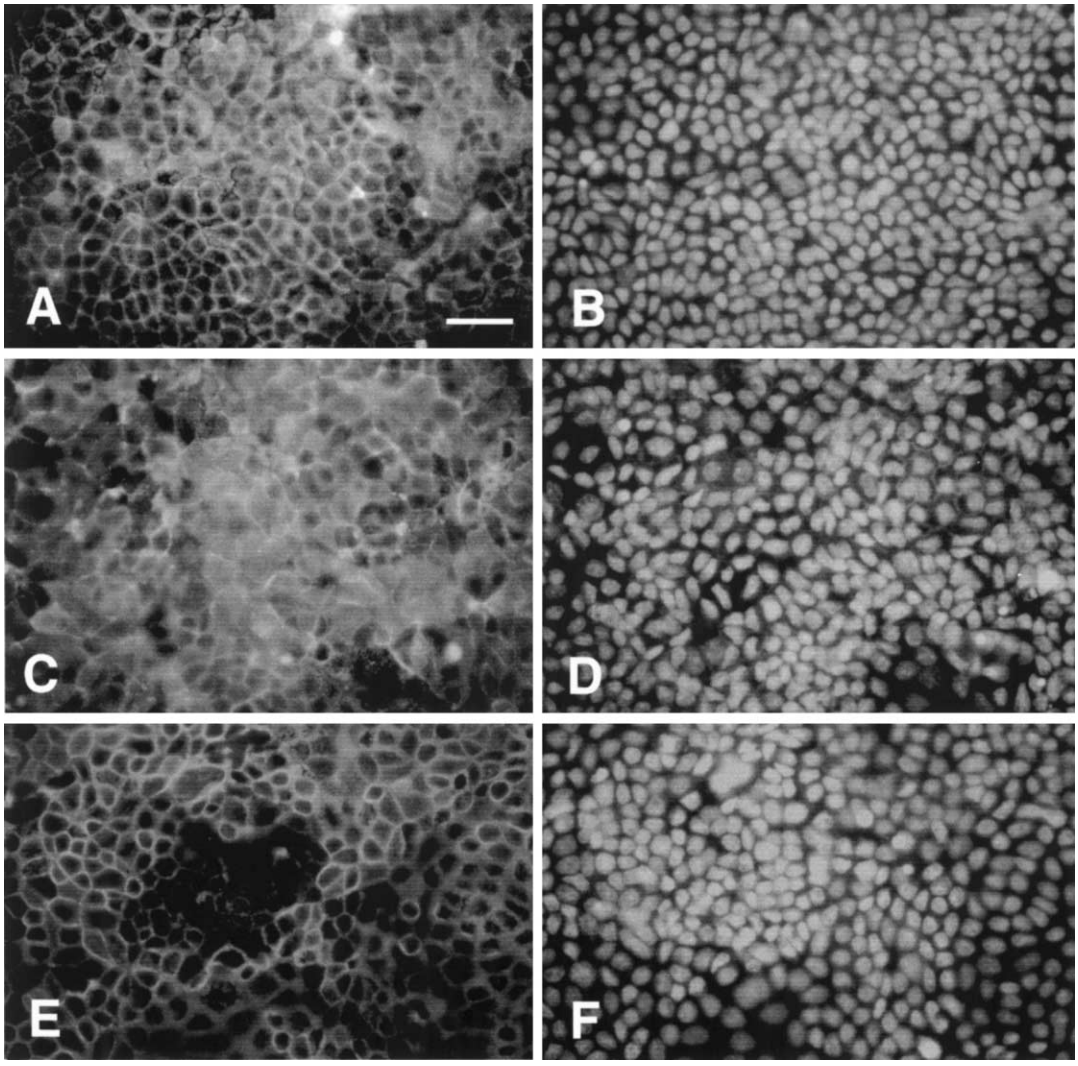

Plate 1. Epifluorescence microscopy of F-actin localized by FITC-phalloidin binding (A, B and C) and corresponding double staining of nuclei with propidium iodide (D, E and F) in control cells (A and D) and in cells treated with 50 μM $FeSO_4$ (B and E) or 50 μM $CuCl_2$ (C and F) for 3 h from the AP side. Magnification is the same in all panels. The bar in panel A corresponds to 35 μm.

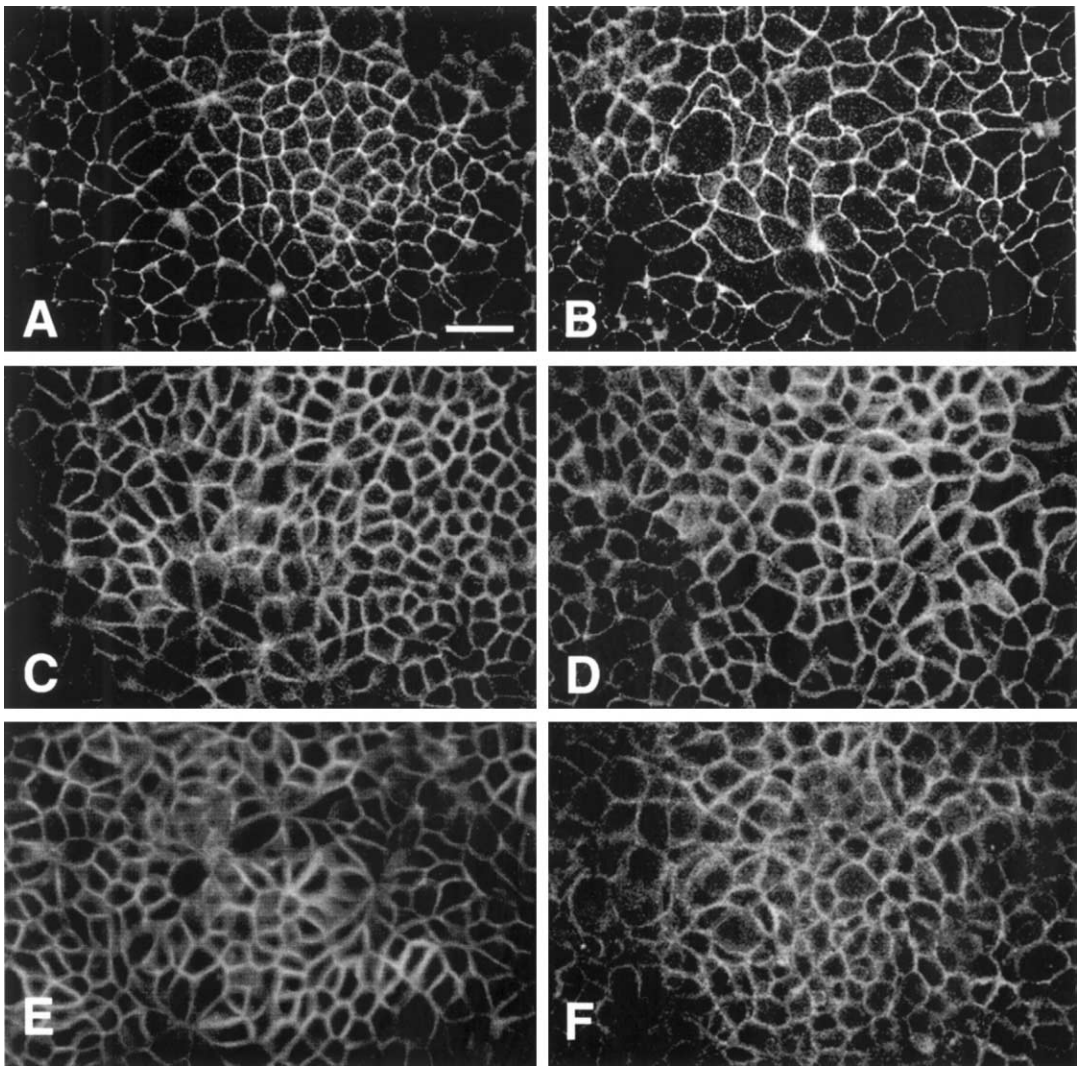

Plate 2. Immunofluorescence of the tight junctional proteins ZO-1 (A and B) and occludin (C and D) and of the adherens-junction protein E-cadherin (E and F) in control Caco-2 cells (A, C, and E) and in cells treated with 100 μM Fe(II) for 3 h from the AP side (B, D and F). Magnification is the same in all panels. The bar in panel A corresponds to 35 μm.

2001). It remains to be determined whether the changes induced by Fe(II) treatment could be ascribed to similar changes to the F-actin cytoskeleton. In addition, the lack of recovery observed following Fe(II) treatment points to some irreversible modification inside the cell or to a cascade of events eventually leading to cell death.

Data in the literature indicate that changes in tight junction permeability produced by a variety of factors and almost invariably associated with derangement of the F-actin cytoskeleton, often but not always, affecting the localization of junctional proteins, are mediated by very different mechanisms. Such mechanisms may sometimes be discriminated by their reversibility and by the kinetics and characteristics of such recovery. The hyperpermeability of Caco-2 tight junctions produced by cytochalasin B or by ethanol treatment, accompanied by F-actin fragmentation and condensation and mediated by activation of myosin light chain kinase, was rapidly reversible (within 2 h) in the absence of de novo protein synthesis (Ma et al., 1995, 1999, 2000). Conversely, the increase in tight junction permeability produced by $CuCl_2$ (Ferruzza et al., 1999b) or by the polycation chitosan chloride (Dodane et al., 1999), was associated in both cases with alterations in the F-actin cytoskeleton, and required a longer time (24 h) as well as RNA and protein synthesis for full recovery. It is unfortunate that none of the reports of hyperpermeability of tight junctions in Caco-2 cells following oxidative damage have addressed the issue of reversibility of the damage (Baker et al., 1995; Rao et al., 1997, 2000; Unno et al., 1997; Banan et al., 2000a,b, 2001; Atkinson and Rao, 2001). The effects of Fe(II) treatment of Caco-2 cells reported in the present work may well involve the production of ROS and/or depletion of GSH from reduction by glutathione peroxidase of H_2O_2 produced

by redox cycling of iron (Halliwell and Gutteridge, 1986).

In conclusion, AP treatment of differentiated filter-grown Caco-2 cells with Cu(II) and Fe(II) at pH 6.0 led to a similar increase in tight junction permeability as monitored by a decrease in TEER. Distinct effects at the level of the F-actin cytoskeleton accompanied such functional changes to the permeability of the cell monolayer. In particular, Cu(II) treatment led to depolymerization of F-actin, while Fe(II) resulted in disorganization of F-actin with loss of the perijunctional ring. These results indicate that different mechanisms are responsible for the hyperpermeability of Caco-2 tight junctions produced by Cu(II) and Fe(II), that in the case of iron may involve oxidative changes to cellular proteins that remain to be clearly elucidated.

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