Oxidant-induced disruption of intestinal epithelial barrier function: role of protein tyrosine phosphorylation


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Rao, R. K., R. D. Baker, S. S. Baker, A. Gupta, and M. Holycross. Oxidant-induced disruption of intestinal epithelial barrier function: role of protein tyrosine phosphorylation. Am. J. Physiol. 273 (Gastrointest. Liver Physiol. 36): G812–G823, 1997.—The effect of hydrogen peroxide (H2O2) on intestinal epithelial barrier function was examined in Caco-2 and T84 cell monolayers. H2O2 reduced transepithelial electrical resistance (TER) of Caco-2 and T84 cell monolayers. This decrease in TER was associated with a decrease in dilution potential and an increase in [3H]mannitol permeability, suggesting an H2O2-induced disruption of the paracellular junctional complexes. H2O2 administration also induced tyrosine phosphorylation of several proteins (at the molecular mass ranges of 50–90, 100–130, and 150–180 kDa) in Caco-2 cell monolayers. Phenylarsine oxide and sodium orthovanadate, inhibitors of protein tyrosine phosphatase, decreased TER and increased mannitol permeability and protein tyrosine phosphorylation (PTP). A low concentration of sodium orthovanadate also potentiated the effect of H2O2 on TER, dilution potential, mannitol permeability, and PTP. Pretreatment with genistein (30–300 µM) and tyrphostin (100 µM) inhibited the effect of H2O2 on TER, dilution potential, mannitol permeability, and PTP. These studies show that H2O2 increases the epithelial permeability by disrupting paracellular junctional complexes, most likely by a PTP-dependent mechanism.

METHODS

Cell culture. Caco-2 cells obtained from American Type Culture Collection (Rockville, MD) were maintained under standard cell culture conditions at 37°C in Dulbecco’s modified Eagle’s medium (DMEM) containing 20% (vol/vol) fetal bovine serum. Cells were grown on polycarbonate membranes in Transwell filters (0.5 mm, Costar, Cambridge, MA). Experiments were performed on the 12th or 13th day after seeding cells onto Transwell filters. Under these conditions, confluent monolayers attained steady-state resistance to passive transepithelial ion flow, and neighboring cells were adjoined by circumferential intercellular tight junctions that restrict the passive flow of ions and solutes, as described previously (5, 19, 27). T84 cells were grown similarly in a mixture (1:1) of DMEM and Ham’s F12 nutrient mixture containing 6% fetal bovine serum, N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (15 mM), and antibiotics (penicillin, ampicillin, and streptomycin).

Oxidant and other treatments. Monolayers were bathed with phosphate-buffered saline (PBS)/bovine serum albumin (BSA) (Dulbecco’s saline containing 1.2 mM CaCl2, 1 mM MgCl2, and 0.6% BSA, 0.2 and 1.0 ml to apical and basal wells, respectively. After 1 h equilibration in PBS/BSA, H2O2 was administered to the apical or basal medium in 10 µl aliquots to achieve a final concentration of 0.5, 1, 2, 5, or 10 mM. In some experiments, a mixture of xanthine oxidase (20 mM) and xanthine (0.25 mM) was administered to apical and basal compartments. Genistein (30–300 µM), genistin (100 µM), or tyrphostin (50 µM) was administered to both the apical and basal media 30 min before H2O2 administration, whereas in others, sodium orthovanadate (vanadate) (0.1 mM) was coadministered with H2O2. Bumetanide (20 µM), phenylarsine oxide (PAO) (30–300 µM), or vanadate (0.1–10 µM) was added to the apical and/or basal media to test the effects on paracellular permeability.

Measurement of TER. Transepithelial electrical resistance (TER) was measured, according to the method of Hidalgo et al. (20), using a Millicell-ERS electrical resistance system (Millipore, Bedford, MA), and calculated as Ω·cm2 by multiplying it with the surface area of the monolayer (0.33 cm2).

Lactate dehydrogenase assay. Cell death was assessed by measuring lactate dehydrogenase (LDH) release. LDH activity was determined, as described by Benford and Hubbard (2),...
in postexperimental apical buffer and cells solubilized in 0.1% Triton X-100 from control and experimental monolayers, and activity released to apical buffer is expressed as percentage of total cellular activity. The percent activity released is the activity in the apical buffer divided by the activity retained in cells plus the activity in the apical buffer Triton X-100. The release of LDH activity into apical buffer in monolayers exposed to 0.1% Triton X-100 at the apical surface for 30 or 60 min was also measured as a positive control.

Unidirectional fluxes of $^{22}\text{Na}^+$ and $[^3\text{H}]$mannitol. Cell monolayers in Transwell filters were incubated under different experimental conditions in the presence of 0.2 $\mu$Ci/ml of $[^2\text{H}]$mannitol (15 Ci/mmol; ICN Biomedicals, Costa Mesa, CA) in the basal well. At different times after $\text{H}_2\text{O}_2$ administration, 100 µl each of apical and basal media were withdrawn and radioactivity 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 square centimeter of surface area. In a separate experiment, 0.1 µCi/ml of $^{22}\text{Na}^+$ (1,200 mCi/mg; DuPont-NEN Research Products, Boston, MA) was administered along with $[^3\text{H}]$mannitol to compare the permeability of $\text{Na}^+$ and mannitol with decrease in TER during the time course of $\text{H}_2\text{O}_2$ effect.

Measurement of dilution potential. Twenty percent sodium chloride dilution potential was measured, as previously described by Madara et al. (23). At the end of the experimental treatments, monolayers were washed once with PBS/BSA and bathed in fresh PBS/BSA, 0.2 and 1.0 ml to apical and basal compartments, respectively. Transepithelial potential difference was recorded using Millicell-ERS electrical resistance system. Twenty percent dilution in the apical compartment was developed by replacing 40 µl of apical medium with 40 µl PBS/BSA in which sodium chloride was replaced with equally osmolar mannitol. The potential difference was recorded again, and the dilution potential was calculated from the difference between the initial potential difference and the potential difference recorded after 20% dilution of apical sodium chloride.

Protein tyrosine phosphorylation. Immediately after the experimental treatment, monolayers were washed with 2 ml of cold PBS without BSA, and monolayers were then lysed in 100 µl monolayer of hot lysis buffer (0.05 M tris(hydroxymethyl)aminomethane, pH 8.0, containing 1% sodium dodecyl sulfate (SDS), 0.1 mM vanadate, and 0.1 mM phenylmethylsulfonyl fluoride). Cell lysate was then heated at 100°C for 5 min and homogenized by passing it 10 times through a 26-gauge needle. The homogenate was centrifuged at 5,000 g for 5 min, and the supernatant was used for Western blotting. The protein content of the supernatants was analyzed by the bicinchoninic acid method (Pierce, Rockford, IL).

Proteins were separated by SDS-polyacrylamide gel electrophoresis on 7.5% gel and transferred to nitrocellulose polyvinylidene difluoride membranes. Phosphotyrosyl proteins were detected by blotting with horseradish peroxidase (HRP)-conjugated anti-phosphotyrosine antibodies and staining with the enhanced chemiluminescence (ECL) method. Protein tyrosine phosphorylation was measured by phosphorylation of intracellular calcium concentration. In this study, Caco-2 cells were grown to confluence on plastic cell culture plates (35 mm; Corning Glass Works, Corning, NY) and experiments were performed at 2–4 days after confluence. Cells were first loaded with fura 2-acetoxymethyl ester (AM) (4 µM) in PBS/BSA at room temperature for 45 min. Intracellular fluorescence was quantitatively monitored using a Zeiss Attofluor digital fluorescence microscope. Intracellular fields from 15 different cells were selected for fluorescence measurements. Emission ratio of 340:380 nm was computed for the intracellular calcium concentration ([Ca$^{2+}$]i). Fura 2 uptake was confirmed by ionomycin administration. $\text{H}_2\text{O}_2$ (10–3,000 µM) was administered after monitoring the baseline [Ca$^{2+}$]i for 2 min or until stable [Ca$^{2+}$]i was achieved.

Fig. 1. $\text{H}_2\text{O}_2$-induced decreases in transepithelial electrical resistance (TER) in Caco-2 cell monolayers (13 days after seeding on to Transwell filters). Values are means ± SE (n = 12 for each group in A and 6 in B-D). A: time course of effects of 0.5 (C), 1.0 (○), 5.0 (△), or 10(△) mM $\text{H}_2\text{O}_2$ on TER of Caco-2 cell monolayers. TER was measured at different times after basal application of $\text{H}_2\text{O}_2$ and expressed as %corresponding baseline TER. Baseline TER was 223±6 Ω·cm$^2$ for 10-, 12-, 14-, and 15-day-old monolayers, respectively. Transepithelial potential difference in Caco-2 cell monolayers was monitored at different times over next 3 h. Baseline values varied from 220±6 to 231±20 Ω·cm$^2$.
At the end of H₂O₂ treatment, cells were tested for fura 2-AM uptake by ionomycin administration. Effect of ethanol (5%) on [Ca²⁺]i was also examined as a positive control.

Chemicals. Genistein was purchased from Calbiochem (San Diego, CA). Bumetanide, tyrphostin-25, PAO, H₂O₂, vanadate, ionomycin, and all other chemicals were of analytical grade and purchased from Sigma Chemical (St. Louis, MO). Mouse monoclonal anti-phosphotyrosine-HRP antibodies were purchased from Transduction Laboratories (Lexington, KY). ECL peroxidase staining kit was from Amersham (Buckinghamshire, UK). Fura 2-AM was purchased from Molecular Probes (Eugene, OR).

Statistics. Comparison between two groups was made by Student’s t-test for grouped data or by analysis of variance and Fisher’s post hoc test for comparisons of more than two groups. The significance in all tests was derived at the 95% or greater confidence level.

RESULTS

The baseline TER of Caco-2 cell monolayers (on day 12 or 13 postseeding) varied from 200 to 350 Ω·cm². Incubation of these monolayers in PBS/BSA did not alter TER at least up to 6 h. H₂O₂ administration to the basal surface at final concentrations of 0.5–10 mM resulted in a concentration-related and time-dependent decrease in TER (Fig. 1A). A significant 15% decrease in TER was seen as early as 30 min after H₂O₂ (10 mM) administration, and a nearly 55% decrease was achieved by 2 h. A 2-h treatment with H₂O₂ (10 mM) produced no change in LDH release into the incubation medium, whereas LDH release was significantly increased by 10.220.33.6 on June 26, 2017 http://ajpgi.physiology.org/ Downloaded from http://ajpgi.physiology.org/
the administration of 0.1% Triton X-100 (Fig. 2). When 
H₂O₂ (10 mM) was withdrawn after 30 min, the TER of 
monolayers continued to decrease at a rate similar to 
that in monolayers in which H₂O₂ was not withdrawn 
(Fig. 1B). The continued decrease in TER after the 
withdrawal of the first dose of H₂O₂ was significantly 
greater when a new dose of H₂O₂ (10 mM) was intro-
duced (Fig. 1B). The H₂O₂-induced decrease in TER 
was greater in 10-day-old monolayers, but the magni-
tude of this effect decreased with further aging of 
monolayers (Fig. 1C). The effects of different doses of 
H₂O₂ (0.5–10 mM) on the TER of Caco-2 cell monolay-
ers could not be reversed by the removal of H₂O₂ and 
continued incubation in PBS/BSA (Fig. 1D).

Control monolayers showed a 20% sodium chloride 
dilution potential by changing the potential difference 
from 20.5 ± 0.05 to 4.3 ± 0.2. H₂O₂ administration 
reduced the dilution potential of Caco-2 cell monolayers 
in a time- and dose-dependent manner (Fig 3A); a 
significant reduction was achieved by 30 min of expo-
sure. The rate of unidirectional flux of [³H]mannitol 
was negligibly low in control monolayers, and it did not 
change during 150-min incubation in PBS/BSA (Fig. 3B). Administration of H₂O₂ (10 mM) produced no effect 
on unidirectional flux of [³H]mannitol until 90 min, but 
it was significantly increased threefold at 120 min and 
ninefold at 150 min. Regression analyses of the relation-
ship between the decrease in TER and dilution poten-
tial or mannitol flux showed a linear relationship 
between TER and dilution potential (Fig. 3C) and a 
curvilinear relationship between TER and mannitol 
flux (Fig. 3D). Administration of bumetanide (20 µM) 
produced no significant effect on TER of Caco-2 cell 
monolayers, and it failed to affect H₂O₂-induced de-
crease in TER (Fig. 4A). Data from dual fluxes of ²²Na 
and [³H]mannitol in Caco-2 cell monolayers at different 
times after H₂O₂ administration are summarized in 
Fig. 4B. Unidirectional flux of ²²Na⁺ increases during
the early phase of the H$_2$O$_2$ effect and showed a linear relationship with the decrease in TER, whereas the increase in [3H]mannitol flux was detected only when the decrease in TER was 40% of baseline values.

Administration of H$_2$O$_2$ (5 mM) to the apical compartment decreased TER of Caco-2 cell monolayers in a concentration- and time-dependent manner (Fig. 5, A and B). Apical H$_2$O$_2$ also reduced dilution potential and increased mannitol permeability of Caco-2 cell monolayers in a concentration-related manner (Fig. 5, C and D). Apical H$_2$O$_2$ was more potent than basal H$_2$O$_2$ in altering TER (Fig. 5B), dilution potential (Fig. 5C), and mannitol permeability (Fig. 5D), and the effects were cumulative when H$_2$O$_2$ was administered to both the apical and basal compartments. Administration of a mixture of xanthine oxidase (20 mU/ml) and xanthine (0.25 mM), a H$_2$O$_2$ generator, also reduced TER and dilution potential of Caco-2 cell monolayers (Fig. 6). This effect of xanthine oxidase plus xanthine was associated with an increase in mannitol permeability (Fig. 6B).

H$_2$O$_2$ stimulated tyrosine phosphorylation of several proteins in Caco-2 cell monolayers (Fig. 7). This effect of H$_2$O$_2$ was concentration (Fig. 7A) and time (Fig. 7B) dependent. The prominent tyrosine-phosphorylated proteins included those in the molecular mass range of 50–90, 100–130, and 150–180 kDa. Once again, apical H$_2$O$_2$ was more potent in stimulating protein tyrosine phosphorylation, and the effects appeared to be cumulative (Fig. 7A). Both apical (5 mM) and basal (10 mM) H$_2$O$_2$ induced protein tyrosine phosphorylation as early as 15 min with peak phosphorylation at 30 min for apical and 120 min for basal H$_2$O$_2$ (Fig. 7B).

PAO, an oxidant and inhibitor of protein tyrosine phosphatase (PTPase), reduced TER (Fig. 8A) and dilution potential (Fig. 8B), which was associated with the increases in mannitol flux (Fig. 8C) and protein tyrosine phosphorylation (Fig. 8D) in Caco-2 cell monolayers; these effects of PAO were concentration related. Vanadate (at 10 mM concentration), a potent inhibitor of PTPase, also produced significant decreases in TER (Fig. 8A) and dilution potential (Fig. 8B) and increases in mannitol permeability (Fig. 8C) and protein tyrosine...
phosphorylation (Fig. 8D). The molecular weight profile of tyrosine phosphorylated proteins was similar to that of H$_2$O$_2$, except that additional tyrosine phosphorylated proteins at the molecular mass range of 35–45 kDa were detected in PAO-treated monolayers. Apical PAO was slightly more potent than basal PAO in increasing paracellular permeability (Fig. 9, A and B). Vanadate, on the other hand, was more potent from the basal surface (Fig. 9, C and D). A low concentration of vanadate (0.1 mM), potentiated the effects of apical H$_2$O$_2$ (1 mM) and basal H$_2$O$_2$ (5 mM) on TER (Fig. 10A), dilution potential (Fig. 10B), and mannitol permeability (Fig. 10C). Vanadate also dramatically potentiated the effect of H$_2$O$_2$ on protein tyrosine phosphorylation (Fig. 10D).

Genistein, an inhibitor of tyrosine kinases, alone produced no effect on the basal TER, dilution potential, or mannitol permeability of Caco-2 cell monolayers. However, treatment with genistein (300 µM) 30 min before H$_2$O$_2$ inhibited H$_2$O$_2$-induced changes in TER (Fig. 11A), dilution potential (Fig. 11B), and mannitol permeability (Fig. 11C). Genistein also inhibited pro-
Fig. 10. Effect of vanadate on H$_2$O$_2$-induced increases in paracellular permeability and protein tyrosine phosphorylation in Caco-2 cell monolayers. A–C: Caco-2 cell monolayers treated with or without 0.1 mM vanadate were exposed to H$_2$O$_2$ (1 or 5 mM at apical or basal surface, respectively). TER was measured at 0 and 60 min after H$_2$O$_2$ treatment, and decrease in TER is presented as % corresponding time 0 values. Dilution potential and mannitol flux were measured at 60 min after treatments. Control monolayers received no treatment. Control, open bars; 0.1 mM vanadate, cross-hatched bars; H$_2$O$_2$, hatched bars; vanadate + H$_2$O$_2$, filled bars. Values are means ± SE (n = 6 for each group). * P < 0.05, significantly different from corresponding H$_2$O$_2$ values. D: Western blot analysis of protein tyrosine phosphorylation stimulated by H$_2$O$_2$ and vanadate. Caco-2 cell monolayers treated for 30 min with 0.1 mM vanadate (lane 1), 1 mM apical H$_2$O$_2$ (lane 2), 1 mM apical H$_2$O$_2$ + 0.1 mM vanadate (lane 3), 5 mM basal H$_2$O$_2$ (lane 4), or 5 mM basal H$_2$O$_2$ + 0.1 mM vanadate (lane 5). Proteins extracted from these monolayers were analyzed for phosphotyrosine.

Fig. 11. Effect of genistein on H$_2$O$_2$-induced increases in paracellular permeability and protein tyrosine phosphorylation in Caco-2 cell monolayers. A–C: Caco-2 cell monolayers treated with or without 0.3 mM genistein were exposed to H$_2$O$_2$ (5 or 10 mM at the apical or basal surface, respectively). TER was measured at 0 and 120 min after H$_2$O$_2$ treatment, and TER decrease is presented as % corresponding time 0 values. Dilution potential and mannitol flux were measured at 120 min after treatments. Control monolayers received no treatment. Control, open bars; 0.3 mM genistein, crosshatched bars; H$_2$O$_2$, hatched bars; genistein + H$_2$O$_2$, filled bars. Values are means ± SE (n = 6 for each group). * P < 0.05, significantly different from corresponding H$_2$O$_2$ values. D: Western blot analysis of protein tyrosine phosphorylation stimulated by H$_2$O$_2$ and genistein. Caco-2 cell monolayers treated for 30 min with 5 mM apical H$_2$O$_2$ (lane 1), 5 mM apical H$_2$O$_2$ + 0.3 mM genistein (lane 2), 10 mM basal H$_2$O$_2$ (lane 3), or 10 mM basal H$_2$O$_2$ + 0.3 mM genistein (lane 4). Proteins extracted from these monolayers were analyzed for phosphotyrosine. Genistein alone induced no tyrosine phosphorylation.
tein tyrosine phosphorylation stimulated by apical or basal H₂O₂ (Fig. 11D). This effect of genistein was concentration related (Fig. 12; 30 μM genistein showed only a poor inhibition, whereas 100 μM genistein inhibited 36–42% of the H₂O₂-induced decrease in TER, and 300 μM genistein completely inhibited the H₂O₂ effect. Administration of tyrphostin (a tyrosine kinase inhibitor) also inhibited the H₂O₂-induced decrease in TER (Fig. 12A), increase in mannitol flux (Fig. 12B), and protein tyrosine phosphorylation (Fig. 12C). Genistin, an inactive analog of genistein, failed to inhibit the effects of H₂O₂ (Fig. 12).

Ionomycin administration resulted in a rapid increase in the [Ca²⁺]i in Caco-2 cell monolayers (Fig. 13A). Administration of H₂O₂ (10 or 3,000 μM), vanadate (10 mM), or vanadate (10 mM) plus H₂O₂ (3,000 μM) failed to affect [Ca²⁺]i (Fig. 13, C-F) at least during the 45-min treatment period. However, administration of ethanol (2%) induced a rapid and transient increase in [Ca²⁺]i levels (Fig. 13B).

Administration of xanthine oxidase plus xanthine at apical and basal surfaces or H₂O₂ (10 mM) at apical or basal surfaces resulted in a time-dependent decrease in TER (Fig. 14A) and increase in mannitol flux (Fig. 14B) in T84 cell monolayers, a colon adenocarcinoma cell line that does not differentiate into a villus-like phenotype. This change in TER and mannitol flux was associated with an increase in PTP (Fig. 14C). The effects of xanthine oxidase plus xanthine in T84 cell monolayers were inhibited by the administration of tyrphostin (50 μM).

DISCUSSION

In this study, we show that H₂O₂ causes a disruption of the paracellular junctional complexes, resulting in an increased passive flow of ions and solutes across the Caco-2 cell epithelium. This study also demonstrates that the H₂O₂-induced increase in epithelial permeability requires protein tyrosine phosphorylation in the Caco-2 and T84 cell epithelia. Exposure to H₂O₂ resulted in a reduction of TER of Caco-2 cell monolayers in a dose- and time-dependent manner. An absence of H₂O₂-induced LDH release indicates that the decrease in TER was not caused by cell lysis and a gross disruption of monolayers at least until 2 h.

The suggestion that the effect of H₂O₂ on the intestinal epithelial barrier function may have a physiological or pathophysiological relevance is supported by the following observations: 1) H₂O₂ concentration as low as 0.2 mM, administered to apical and basal surfaces, produced a significant increase in paracellular permeability. Although a significant body of evidence indicates that oxidants play an important role in the development of epithelial injury in different intestinal disorders (12–15, 34, 37), it is difficult to accurately measure the concentration of H₂O₂ under physiological or pathophysiological conditions due to its very short half-life. Additionally, the H₂O₂ concentration in the microenvironment of the epithelial cell may be more important with respect to its potential to cause cellular damage. 2) A 30- to 60-min exposure to H₂O₂ was sufficient to cause the observed epithelial injury, which was not reversible at least for 3 h in PBS/BSA. 3) Repeated administration of H₂O₂ caused more severe injury to the epithelium, indicating that continuous oxidant generation under an in vivo condition may be more toxic than bolus administration under the present in vitro condition. This is supported by the observation that xanthine oxidase plus xanthine also increased paracellular permeability in Caco-2 cell monolayers. Under similar conditions, xanthine oxidase has been shown previously to generate H₂O₂, achieving the steady-state concentration of 80–100 μM (28). 4) H₂O₂-induced decrease in TER was more severe in 10- or 12-day-old monolayers than it was in 14- or 15-day-old monolayers. It is unlikely that the age-dependent stability of Caco-2 cell monolayers is related to the differential abilities of cells to metabolize H₂O₂ as glutathione

Fig. 12. Effect of different concentrations (30–300 μM) of genistein (filled bars), 100 μM genistin (horizontal-striped bars), and 100 μM tyrphostin (crosshatched bars) on H₂O₂ (basal 10 mM)-induced changes in TER (A), mannitol flux (B), and protein tyrosine phosphorylation (C) in Caco-2 cell monolayers. These results compared with those of monolayers treated with H₂O₂ alone (hatched bars) or monolayers with no treatment (open bars). A and B: TER was measured at 0 and 120 min after H₂O₂ treatment, and decrease in TER is presented as % corresponding time 0 values. Mannitol flux was measured at 120 min after treatments. Control monolayers received no treatment. Values are means ± SE (n = 6 for each group). *Significantly (P < 0.05) different from corresponding H₂O₂ values. C: Western blot analysis of protein tyrosine phosphorylation stimulated by H₂O₂ with or without presence of genistein, genistin, or tyrphostin. Cell monolayers treated for 30 min with 10 mM basal H₂O₂ (lane 1), 10 mM basal H₂O₂ + 0.1 mM genistein (lane 2), 10 mM basal H₂O₂ + 0.03 mM genistin (lane 3), 10 mM basal H₂O₂ + 0.1 mM genistin (lane 4), 10 mM basal H₂O₂ + 0.3 mM genistein (lane 5), or 10 mM basal H₂O₂ + 0.1 mM tyrphostin (lane 6). Proteins extracted from these monolayers were analyzed for phosphorysorine. Alone, genistein, genistin, or tyrphostin induced no tyrosine phosphorylation.

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peroxidase activity in this cell does not change after 10 days (1). It is however possible that differentiation of cells into columnar villus-like cells is responsible for such a relative stability of cells against H$_2$O$_2$. Caco-2 cells grow to form a tight monolayer and at confluence they differentiate into villus-like columnar cells (26). Reduced cell membrane permeability to H$_2$O$_2$ and/or expression of protective factors in the differentiated cells may play an important role in this resistance to oxidant injury.

A decrease in TER may reflect an increase in movement of solutes and ions across the epithelium by transcellular and/or paracellular pathways. A reduction of 20% sodium chloride dilution potential by H$_2$O$_2$ treatment suggests a reduction of the charge selectivity of tight junctions. The intestinal epithelial tight junctions are known to selectively impede anions, whereas cations freely diffuse through these junctions (23). A linear correlation between the dilution potential and TER during the time course of H$_2$O$_2$ injury indicates a direct relationship between electrical resistance and charge selectivity of tight junctions. An association of H$_2$O$_2$-induced decrease in TER with an increase in unidirectional flux of [3H]mannitol indicates that H$_2$O$_2$ disrupts the paracellular junctional complexes in this epithelial monolayer. Interestingly, H$_2$O$_2$-induced increase in mannitol flux was observed only after 90 min of exposure, demonstrating a curvilinear relationship between mannitol flux and TER decrease. Unlike mannitol permeability, an increase in Na$^+$ flux was observed during the early phase. A linear relationship between the decrease in TER and Na$^+$ flux indicated that initially, permeability to small molecules may have increased by widening of the junctional pores, however, it was not wide enough to allow mannitol to diffuse through. A possible explanation for the decrease in TER without altering mannitol flux during the early phase is an increase in chloride secretion as it was previously shown that an initial decrease in TER of T84 cell monolayers induced by polymorphonuclear neutrophils was caused by chloride secretion (25). Bumetanide, an inhibitor of the basal Na$^+$-K$^+$-2Cl trans-...
and was concentration related. The mechanism of H$_2$O$_2$-induced stimulation of protein tyrosine phosphorylation in Caco-2 cell monolayers is not clear. It is likely that H$_2$O$_2$ stimulates tyrosine phosphorylation by inhibiting the activity of PTPases as it is known that H$_2$O$_2$ is a potent inhibitor of PTPases both in vitro (17) and in vivo (18). H$_2$O$_2$ has been previously shown to induce protein tyrosine phosphorylation in several cells (16, 18, 22). Although the significance of tyrosine phosphorylated proteins in mediating the biological effects of H$_2$O$_2$ is not known, a few studies suggest that H$_2$O$_2$-induced increase in tyrosine phosphorylation of insulin receptors resulting in receptor tyrosine kinase may mediate its insulinomimetic effects in rat hepatoma cells (16, 18) and rat adipocytes (22). Recent studies (4, 31, 36) have indicated that protein tyrosine phosphorylation may play a role in the function of adherens junctions and tight junctions of epithelial tissue. Although apical H$_2$O$_2$ was more potent in inducing protein tyrosine phosphorylation than basal H$_2$O$_2$, the molecular weight profiles of tyrosine phosphorylated proteins appear similar when H$_2$O$_2$ was administered to apical or basal compartments. The time course of phosphorylation indicates that a peak phosphorylation was achieved by apical H$_2$O$_2$ at 30 min followed by a gradual decrease until 180 min. Peak phosphorylation did not occur until 120 min in basally stimulated cells, indicating a difference between apical and basal H$_2$O$_2$ in inducing protein tyrosine phosphorylation.

In the present study, the relationship between H$_2$O$_2$-induced protein tyrosine phosphorylation and paracellular permeability was determined by evaluating the effect of PTPase inhibitors (PAO and vanadate) on epithelial permeability in control monolayers and tyrosine kinase inhibitor (genistein) on H$_2$O$_2$-induced increase in epithelial permeability. PAO, a PTPase inhibitor, reduced TER and dilution potential and increased mannitol permeability of Caco-2 cell monolayers. PAO-induced increase in paracellular permeability was associated with an increase in protein tyrosine phosphorylation of several proteins. The electrophoretic profile of tyrosine phosphorylated proteins was very similar to that of H$_2$O$_2$-treated monolayers except that PAO phosphorylated additional proteins at the molecular mass range of 35–45 kDa. Vanadate (a well-established PTPase inhibitor) at a concentration of 10 mM, but not of 1 mM, produced an increase in permeability and protein tyrosine phosphorylation. A low concentration of vanadate (0.1 mM) however produced a strong potentiation of the effect of a low H$_2$O$_2$ concentration on paracellular permeability and protein tyrosine phosphorylation. All these observations clearly indicate an association between an increase in paracellular permeability and protein tyrosine phosphorylation. The low potency of vanadate by itself contrasts with the known inhibitory effects of vanadate on PTPase activity in vitro (32). The reason for this low potency of vanadate in Caco-2 cell monolayer is possibly due to a poor penetration of vanadate into Caco-2 cells. This suggestion is supported by the previous observations that either prolonged incubation or a very high concentration of vanadate was necessary to induce a biological effect in 3T3 L1 adipocytes (8), rat hepatocytes (30), and IM-9 lymphocytes (33). The dramatic potentiation of the H$_2$O$_2$ effect on both paracellular permeability and protein tyrosine phosphorylation by 0.1 mM orthovanadate can be explained by the H$_2$O$_2$-induced oxidation of orthovanadate into pervanadate (21), which may facil-
H2O2-induced increases in paracellular permeability are supported by the experiments showing similar inhibition of paracellular permeability. This observation is supported by the lack of influence on Ca^{2+} by vanadate or vanadate plus H2O2.

The present study also demonstrates a similar phenomenon in T84 cell monolayers, another colon adenocarcinoma cell line that grows to form a tight monolayer of cells. Unlike Caco-2 cells, these cells do not differentiate into villus-like enterocytes, rather they possess properties similar to crypt cells. Both xanthine oxidase plus xanthine and H2O2 administration decreased TER and dilution potential and increased mannitol flux and PTP in T84 cell monolayers. These effects of oxidants in T84 cell monolayers were prevented by tyrphostin administration. Similar effects of oxidants in two different intestinal cell lines suggest that this action of oxidants occurs generally in the intestinal epithelium. However, the fact that Caco-2 and T84 cells are transformed cells raises the possibility that the effect of oxidants in a nontransformed cell may not be similar. However, the transformed cells are known to be resistant to oxidant injury, and therefore, the oxidant-induced epithelial damage may be more pronounced than the damage observed in this study.

In summary, these studies show that H2O2 induces a disruption of the paracellular junctional complexes of the Caco-2 epithelium, which is mediated by a rapid induction of protein tyrosine phosphorylation.


