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ORIGINAL ARTICLE

Curcumin Ameliorates Hydrogen Peroxide-Induced Epithelial Barrier Disruption by Upregulating Heme Oxygenase-1 Expression in Human Intestinal Epithelial Cells

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Abstract
Background Disruption of epithelial tight junctions (TJ) followed by loss of barrier function is of crucial importance in the pathogenesis of a variety of gastrointestinal disorders. Heme oxygenase-1 (HO-1), which can be induced by curcumin (Cur), provides protection against various forms of oxidative stress.
Aims The protective effect of Cur on oxidative stress-induced intestinal barrier disruption in human intestinal epithelial cells was elucidated in this study.
Methods H_2O_2-induced Caco-2 enterocytic monolayers were incubated in the presence or absence of Cur and/or zinc protoporphyrin (ZnPP). The trans-epithelial electrical resistance (TEER) and the flux of sodium fluorescein in the filter-grown Caco-2 cell monolayers were measured. The expression and localization of the TJ protein occludin and zonula occluden-1 (ZO-1) were evaluated by western blot and immunofluorescence microscopy. The mRNA and protein levels of HO-1 were analyzed by real-time PCR and western blot.
Results Cur attenuated H_2O_2-induced disruption of paracellular permeability (TEER 52.02 ± 10.15% vs 22.71 ± 3.11%; sodium fluorescein flux 12.41 ± 2.19% vs 32.00 ± 4.97%, $P < 0.05$) and induced HO-1 mRNA (6.64 ± 0.48 vs 3.22 ± 0.28, $P < 0.05$) and protein (291.00 ± 9.17% vs 99.00 ± 10.00%, $P < 0.05$) expression in Caco-2 cells. After administration of H_2O_2, occludin and ZO-1 proteins were restored by Cur (occludin 175.67 ± 29.50% vs 53.67 ± 24.19%, $P < 0.05$; ZO-1 139.67 ± 33.71% vs 36.00 ± 15.88%, $P < 0.05$) and this effect was blocked by HO-1 inhibitor, ZnPP (occludin 54.67 ± 10.02% vs 168.33 ± 36.47%, $P < 0.05$; ZO-1 50.00 ± 15.13% vs 117.67 ± 38.81%, $P < 0.05$).
Conclusion Cur protects human intestinal epithelial cells against H_2O_2-induced disruption of TJ and barrier dysfunction via the HO-1 pathway.

Keywords Curcumin · Intestinal mucosal barrier · Tight junction · Heme oxygenase-1 · Oxidative stress

Abbreviations

TJ	Tight junction
OJ	Obstructive jaundice
IBD	Inflammatory bowel disease
AP	Acute pancreatitis
Cur	Curcumin
AJ	Adhere junctions
JAM	Junctional adhesion molecule
ZOs	Zonula occludens
H_2O_2	Hydrogen peroxide
HO-1	Heme oxygenase-1
DMEM	Dulbecco's Vogt modified Eagle's media
TEER	Trans-epithelial electrical resistance
ZnPP	Zinc protoporphyrin
LDH	Lactate dehydrogenase
MDA	Malondialdehyde

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Introduction

The gastrointestinal epithelium is a highly selective barrier that normally prevents passage of harmful molecules across the mucosa and into the circulation [1]. The effectiveness and stability of this epithelial barrier depend on the activity of junctional complexes, for example tight junctions (TJ) [1, 2]. TJ are among the most important components of the junctional complex and seal epithelial cells together in a way that prevents even small molecules from leaking between cells [3, 4]. Studies reported during the past decade have shown that TJ are composed of at least three types of transmembrane proteins: occludin, claudins, and junctional adhesion molecule (JAM). Transmembrane proteins interact with other intracellular plaque proteins including zonula occludens (ZO-1, 2, and 3), which anchor the transmembrane proteins to the actin cytoskeleton [3, 4]. A significant body of evidence indicates that disruption of TJ and increased paracellular permeability are crucially important in the pathogenesis of gastrointestinal disorders, for example inflammatory bowel disease (IBD), obstructive jaundice (OJ), acute pancreatitis (AP), alcohol-induced liver injury, among others [1, 2, 5–7]. Oxidative stress-induced opening of the intestinal TJ barrier is an important mechanism contributing to the TJ barrier defect present in a variety of conditions of the gut [6, 8–10].

Caco-2, a human intestinal epithelial cell line, is derived from a human colonic adenocarcinoma and spontaneously differentiates in culture into cells with some characteristics of the enterocyte, with expression of the corresponding biochemical marker [11]. The Caco-2 cell is the most popular in-vitro cell line for prediction of transport of drugs [12]. The availability of cell-culture models based on a human cell line with the characteristics of the mature enterocyte, the typical cell of the small intestine, offers the possibility of studying and predicting in-vivo intestinal epithelial barrier integrity and function [9–11, 13–15].

Hydrogen peroxide (H_2O_2), a highly toxic oxidizing agent, is constantly being generated within all types of cell, including colonic epithelial cells, during normal aerobic metabolism and must be quickly detoxified by antioxidant defense enzymes, for example catalase in peroxisomes and glutathione peroxidase in mitochondria. Otherwise, the amount of H_2O_2 would induce cell injury. H_2O_2 at low micromolar levels is poorly reactive in biological systems. However, higher concentrations H_2O_2 can inactivate glyceraldehyde-3-phosphate dehydrogenase, a glycolytic enzyme. It is well established that H_2O_2 is the major oxidant species involved in oxidative stress-induced cell injury, which is associated with increased monolayer permeability, by disrupting TJ, in vitro and in vivo [13–15]. A large number of studies have indicated that H_2O_2 disrupts intestinal epithelial barrier function leading to elevate paracellular permeability [9–15]. In Caco-2 cell monolayers it has been demonstrated that disruption of barrier function by oxidative stress is mediated by H_2O_2 [16].

Heme oxygenase-1 (HO-1), a ubiquitous and redox-sensitive inducible stress protein degrades heme to CO, iron, and biliverdin [17]. It can be induced by a variety of oxidative-inducing agents, including H_2O_2. Recently, numerous in-vitro and in-vivo studies have shown that induction of HO-1 is an important cellular protective mechanism against oxidative injury [16–18]. Curcumin (Cur) is a major active component of the food flavor turmeric, isolated from the powdered dry rhizome of *Curcuma longa* Linn. The component has a variety of pharmacological activity, including anti-oxidative, anti-inflammatory, anti-carcinogenic, anti-diabetic, and anti-HIV effects [18–20]. Cur is a naturally occurring compound that has been identified as a potent inducer of HO-1 [18, 21]. For this reason, great interest has been generated in investigating the HO-1-inducing potency of Cur and the protective effect of Cur on H_2O_2-mediated TJ disruption in human intestinal epithelial cells.

In this study, we evaluated the ability of Cur to induce HO-1 and the protective effect of Cur on H_2O_2-induced TJ disruption and barrier dysfunction in human intestinal epithelial cells. This will provide a rationale to develop Cur for possible treatment of intestinal barrier disruption diseases.

Methods

Cell Culture and Treatment

Caco-2 cells were grown in high-glucose Dulbecco's Vogt modified Eagle's media (DMEM) with 10% fetal calf serum. Cells were maintained under a humidified atmosphere of 5% CO_2 in air in an incubator at 37°C. Caco-2 cells were then grown on polycarbonate membranes in transwell inserts (6.5 mm; Costar, Cambridge, MA, USA) for four weeks to form cell monolayers.

Cur was dissolved in dimethyl sulfoxide (DMSO) as vehicle. After pretreatment with Cur for 30 min, Caco-2 cell monolayers were incubated in the medium control or treated with H_2O_2 (500 μM) plus 0, 5, 20, 80 μM Cur (Sigma, St Louis, MO, USA) for 6 h. In some experiments, cells were pretreated with ZnPP (Sigma), a specific inhibitor of HO-1, for 12 h before Cur. Caco-2 cell monolayers were split into the following groups: control (1‰ DMSO) group, H_2O_2 (500 μM) group, H_2O_2 + Cur (5, 20, and 80 μM) groups, H_2O_2 + Cur (20 μM) + ZnPP (20 μM) group, H_2O_2 + ZnPP (20 μM) group. Culture medium, protein and mRNA were harvested from H_2O_2-induced Caco-2 exposed to 20 μM Cur for 1, 3, 6 h and also different concentrations of Cur.

Measurement of Trans-Epithelial Electrical Resistance (TEER)

TEER was measured by use of Millcell-ERS (Millipore, Bedford, MA, USA) and calculated as ohms × cm^2 (Ω cm^2) by multiplying it by the surface area of the monolayer (0.33 cm^2). TEER recorded in unseeded Transwell inserts (approximately 30 Ω cm^2) was subtracted from all values. Basal TEER varied from 250 to 350 Ω cm^2. Changes in TEER during experimental conditions were calculated as the percentage of corresponding basal values. Duplicate cell monolayers were used for each group in each experiment, and the experiment was repeated at least four times.

Unidirectional Flux of Sodium Fluorescein Assay

The permeability was estimated by measuring the paracellular transport of fluorescein sodium (Sigma) of MW 376 Da and final concentration 67 mg/ml in the basal well [22]. Six hours after experimental treatment, 100 µl was withdrawn from each apical well and the fluorescence was measured in a microplate fluorescence reader (FLX-800 fluorescence microplate reader; Bio TEK Instruments, Winooski, VT, USA) with excitation at 490 nm and emission at 520 nm. The flux of fluorescein sodium in the apical well was calculated as the percentage of total fluorescence administered into the basal well per hour per cm^2 surface area.

Lactate Dehydrogenase (LDH), Malondialdehyde (MDA), and Superoxide Dismutase (SOD) Determination

LDH, MDA, and SOD in the culture medium were measured using detection kits (Jiancheng Institute of Biotechnology, Nanjing, China) in accordance with the manufacturer's instructions. All experiments were performed in triplicate.

Real-Time PCR

Total RNA was isolated from cells by Trizol isolation (Invitrogen, Carlsbad, CA, USA), in accordance with the manufacturer's recommendations, and quantified spectrophotometrically (260 nm). One microgram of total RNA was reverse transcribed by use of the PrimeScript RT reagent Kit (DRR037S; TaKaRa Bio, Japan). cDNA was then amplified by use of the SYBR Premix Ex Taq Kit (DRR041S; TaKaRa Bio) on an ABI Prism 7300 sequence detection PCR system (Applied Biosystems, USA). Briefly, the 20-µl reaction system contained 10 µl of SYBR green PCR Master Mix, 0.8 µM primers, and diethyl pyrocarbonate water. The primer sequence was designed by use of PrimerQuest (Integrated DNA Technologies, Cambridge, MA, USA). The primer sequences for HO-1 and GAPDH were: HO-1, forward 5′-CTCTTGGCTGGCTTCCTT-3′, reverse 5′-GGCTCCTTCCTCCTTTCC-3′; GAPDH, forward 5′-ACTTTGGTATCGTGGAAGGACT-3′; reverse 5′-GTAGAGGCAGGGATGATGTTCT-3. Optimum conditions for PCR amplification of cDNA were established by use of routine methods. The Ct values of the house-keeping gene and the assayed gene ranged from 15 to 25 and melting curves were determined to show primer specificity. Relative mRNA levels were calculated after normalization to GAPDH. The result is presented as changes compared with medium control (nontreatment).

Western Blot

Western blot analysis was undertaken by using the following primary antibodies: HO-1 (Stressgen, Victoria, BC, Canada), occludin (Zymed Laboratories, Carlsbad, CA, USA), ZO-1 (Zymed Laboratories), and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (Table 1).

Caco-2 cells were lysed with lysis buffer on ice [23]. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted on to a polyvinylidene fluoride membrane (Millipore). The membranes were washed three times with PBS, and then blocked in 5% (*w*/*v*) skimmed milk in TBS for 2 h at room temperature. Subsequently, membranes were incubated with primary antibodies at 4°C overnight, after washing with PBS three times, incubated with corresponding horseradish-peroxidase conjugated secondary antibodies (1:4,000 in 5% (*w*/*v*) skimmed milk in TBS buffer) for 2 h at room temperature. Immunoreactive proteins were detected using enhanced chemiluminescence (ECL). For protein quantification, bands were analyzed by densitometry, using GAPDH as internal control. Western blot analysis for each experiment was repeated at least three times with similar results. The band density was analyzed by use of NIH ImageJ 1.38 software.

Table 1 Concentration of antibodies

Antibody	Dilution
HO-1 (polyclonal antibody)	1:1,000
Occludin (polyclonal antibody)	1:300
Occludin (monoclonal antibody)	1:250
ZO-1 (polyclonal antibody)	1:250
GAPDH (polyclonal antibody)	1:200
Cy3-labeled Goat anti-mouse IgG	1:250
Fluorescein-conjugated affinipure goat anti-rabbit IgG	1:200

Immunofluorescence Staining of TJ Proteins

Under different experimental conditions, cell monolayers were washed three times with ice–cold PBS and fixed in buffered 4% paraformaldehyde for 20 min. The fixed monolayers were rehydrated in PBS and blocked with 5% bovine serum albumin (BSA) diluted in PBS for 1 h, then stained with primary antibodies (mouse monoclonal anti-occludin and rabbit polyclonal anti-ZO-1 antibodies) overnight at 4°C, and then washed with PBS three times, subsequently incubated with a mixture of secondary antibodies (AlexaFluor 488-conjugated anti-rabbit IgG and Cy3-conjugated anti-mouse IgG) for 1 h at room temperature. Monolayers were embedded on cover slips and viewed with a confocal laser-scanning microscope.

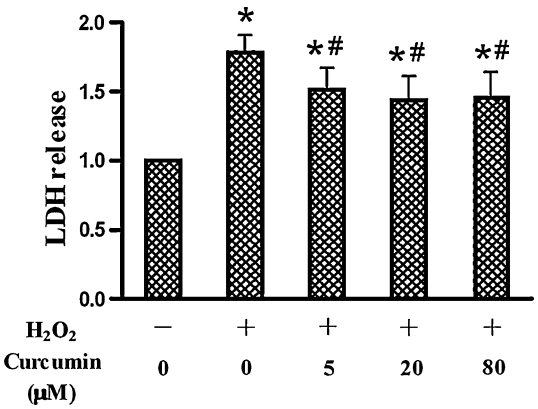

Fig. 1 The protective effects of Cur on H_2O_2-induced cytotoxicity by measuring LDH activity in medium. Caco-2 cells treated with Cur (administered 30 min before H_2O_2) were incubated in the presence or absence of 500 μM H_2O_2 for 6 h. Pretreatment with Cur (5, 20, and 80 μM) attenuated the increased LDH activity induced by H_2O_2. Results are presented as means ± SD (*$P < 0.05$ compared with the control culture, #$P < 0.05$ compared with cells exposed to H_2O_2 alone, $n = 3$)

Statistical Analysis

Results are presented as mean ± SD. The significance of differences between the groups was analyzed by use of one-way analysis of variance followed by LSD. A P value <0.05 was considered statistically significant.

Results

Curcumin (Cur) Attenuated H_2O_2-Induced Oxidative Stress and Cytotoxicity in Caco-2 Cells

Treatment of Caco-2 cell monolayers with 500 μM H_2O_2 for 6 h resulted in cytotoxic effects with dramatic increases in LDH release as determined by the LDH release assay (179.27 ± 11.00% vs 100 ± 0.00%, $P < 0.05$). Pretreatment with Cur (5, 20, and 80 μM) induced cytoprotection against H_2O_2-induced cytotoxicity (151.94 ± 14.56%, 144.12 ± 16.45%, 146.22 ± 17.94% vs 179.27 ± 11.00%, $P < 0.05$), but the protective effects of each concentration were similar ($P > 0.05$; Fig. 1). There was no evidence of cytotoxic effects with Cur at the indicated time point and for these concentrations, as determined by the LDH release assay (data not shown). Treatment of Caco-2 cell monolayers with 500 μM H_2O_2 for 6 h resulted in lipid peroxidation which was observed through formation of MDA (2.91 ± 0.62 nmol/ml vs 0.92 ± 0.14 nmol/ml, $P < 0.05$; Fig. 2a). The levels of MDA concentration in H_2O_2-induced cells were significantly increased compared to control cells which can be blocked by Cur treatment (1.46 ± 0.14 nmol/ml, 1.29 ± 0.19 nmol/ml, 1.71 ± 0.27 nmol/ml vs 2.91 ±0.62 nmol/ml, $P < 0.05$; Fig. 2a). However, the protective effects were similar at the indicated concentrations ($P > 0.05$; Fig. 2a). The protective effect of Cur on H_2O_2-induced SOD activity was further examined (Fig. 2b). H_2O_2 alone inhibited the activity of SOD (5.01 ± 0.24 unit/ml vs 6.71 ± 0.21 unit/ml, $P < 0.05$) but pretreatment with Cur (5, 20, and

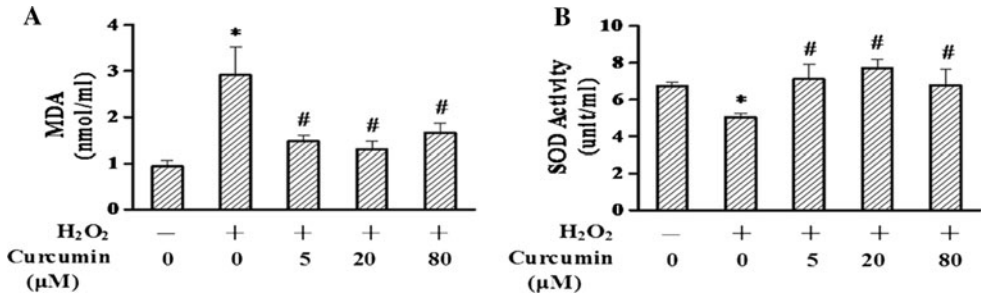

Fig. 2 Effects of Cur on H_2O_2-induced oxidative stress. **a** The protective effects of Cur on H_2O_2-induced lipid peroxidation by measuring MDA. Pretreatment with Cur (5, 20, and 80 μM) attenuated the increased MDA induced by H_2O_2. **b** The protective effects of Cur on H_2O_2-induced SOD activity. Pretreatment with Cur (5, 20, and 80 μM) increased the reduced SOD activity induced by H_2O_2. Caco-2 cells treated with Cur (administered 30 min before H_2O_2) were incubated in the presence or absence of 500 μM H_2O_2 for 6 h. Results are presented as means ± SD (*$P < 0.05$ compared with the control culture, #$P < 0.05$ compared with cells exposed to H_2O_2 alone, $n = 3$)

80 μM) rescued the reduced activity of SOD (7.07 ± 0.83 unit/ml, 7.68 ± 0.51 unit/ml, 6.74 ± 0.92 unit/ml vs 5.01 ± 0.24 unit/ml, $P < 0.05$).

Curcumin (Cur) Protected Caco-2 Cell Monolayers Against H_2O_2-Induced Disruption of Paracellular Permeability

Treatment of Caco-2 cell monolayers with 500 μM H_2O_2 resulted in a significant decrease in TEER time-dependently (100 ± 0.00, 57.98 ± 7.07, 45.25 ± 8.37, 36.17 ± 8.67, 22.71 ± 3.11%; Fig. 3a), and increase in sodium fluorescein permeability (32.00 ± 4.97% vs 6.10 ± 1.43%, $P < 0.05$; Fig. 3b). Pretreatment with Cur partially reversed the effects of H_2O_2-induced hyperpermeability at the times indicated (TEER 100 ± 0.00, 85.76 ± 7.70, 71.51 ± 5.34, 61.79 ± 3.40, 52.02 ± 10.15%; sodium fluorescein flux 12.41 ± 2.19% vs 32.00 ± 4.97%, $P < 0.05$). The protective effect of Cur was partially dose-dependent and the best effect was obtained at 20 μM ($P < 0.05$; Fig. 3a, b). However, incubation of Caco-2 monolayers with 20 μM ZnPP, a specific HO-1 inhibitor, blocks the protective effects of Cur (TEER 23.35 ± 5.82, 52.01 ± 10.15%; sodium fluorescein flux 31.01 ± 2.28% vs 12.41 ± 2.19%, $P < 0.05$; Fig. 3a, b). ZnPP itself did not affect TEER (data not shown).

Curcumin (Cur) Ameliorated H_2O_2-Induced TJ Disruption via HO-1 in Caco-2 Cell Monolayers

Treatment of Caco-2 cells with 500 μM H_2O_2 alone resulted in significant increases of both mRNA (3.22 ± 0.28 vs 1.00 ± 0.00, $P < 0.05$) and protein levels of HO-1 (HO-1/GAPDH 99.00 ± 10.00% vs 46.70 ± 7.20%, $P < 0.05$), which was further potentiated by Cur (5, 20, and 80 μM) pretreatment (5.58 ± 1.06, 6.64 ± 0.48, 5.64 ± 0.88 vs 3.22 ± 0.28, $P < 0.05$; HO-1/GAPDH 198.67 ± 87.96%, 291.00 ± 9.17%, 191.33 ± 37.11% vs 99.00 ± 10.00%, $P < 0.05$). The protective effect of Cur was partially dose-dependent and the best effect was obtained at 20 μM (Figs. 4a, 5a). However, induction of HO-1 mRNA (3.33 ± 0.41 vs 6.64 ± 0.48, $P < 0.05$) and protein expression (HO-1/GAPDH 96.00 ± 25.00% vs 283.69 ± 31.74%, $P < 0.05$) was blocked by ZnPP (Figs. 4b, 5b). After pretreated with 20 μM Cur for 30 min, administration of 500 μM H_2O_2 to Caco-2 cells increased expression of HO-1 at mRNA (4.34 ± 0.39 vs 1.00 ± 0.00, $P < 0.05$) and protein level (HO-1/GAPDH 149.33 ± 6.81% vs 43.67 ± 10.60%, $P < 0.05$) at 1 h and the effect increased with time up to 6 h of treatment (7.25 ± 0.72 vs 1.00 ± 0.00, $P < 0.05$; HO-1/GAPDH 372.33 ± 102.34% vs 43.67 ± 10.60%, $P < 0.05$; Figs. 4c, 5c).

The expression and localization of TJ protein occludin and ZO-1 were measured by western blot and observed by immunofluorescence microscopy. Treatment of Caco-2 cell monolayers with 500 μM H_2O_2 resulted in significant reduction of occludin (occludin/GAPDH 53.67 ± 24.19% vs 178.00 ± 29.51%, $P < 0.05$) and ZO-1 expression (ZO-1/GAPDH 36.00 ± 15.88% vs 133.00 ± 26.46%, $P < 0.05$). However, the effects of H_2O_2-induced reduction of occludin and ZO-1 were attenuated by pretreatment of cells with Cur in a partially dose-dependent manner and the highest activation was observed at 20 μM (occludin/

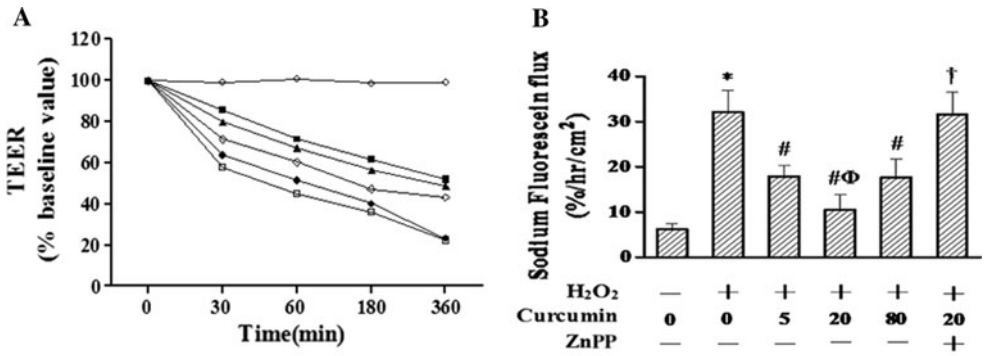

Fig. 3 HO-1 was involved in Cur-mediated prevention of H_2O_2-induced paracellular permeability in Caco-2 cells. **a** Effects of Cur on the H_2O_2-induced reduction in TEER of Caco-2 monolayers. Caco-2 cell monolayers were incubated without (*open circles*) or with 500 μM H_2O_2 in the absence (*open squares*) or presence of 5 μM (*filled triangles*), 20 μM (*filled squares*), 80 μM (*diamond symbols*) Cur that was administered 30 min before H_2O_2. In some groups, cell monolayers were pretreated with ZnPP at 20 μM (*filled circles*) for 12 h before administration of Cur. Treatment of Caco-2 cell monolayers with 500 μM H_2O_2 resulted in a significant decrease in TEER. Pretreatment with Cur partially reversed the H_2O_2-induced decrease in TEER. **b** Effects of Cur on H_2O_2-induced reduction in sodium fluorescein permeability of Caco-2 monolayers. Sodium fluorescein permeability was measured 6 h after H_2O_2 administration. Treatment of Caco-2 cell monolayers with 500 μM H_2O_2 resulted in a significant increase in sodium fluorescein permeability. Pretreatment with Cur partially reversed the H_2O_2-induced increase in sodium fluorescein permeability. Values are presented as means ± SD ($^*P < 0.05$ compared with the control cultures, $^{\#}P < 0.05$ compared with cells exposed to H_2O_2 alone, $^{\Phi}P < 0.05$ compared with cells treated with 5 and 80 μM Cur, $^{\dagger}P < 0.05$ compared with cells treated only with 20 μM Cur, $n = 4$)

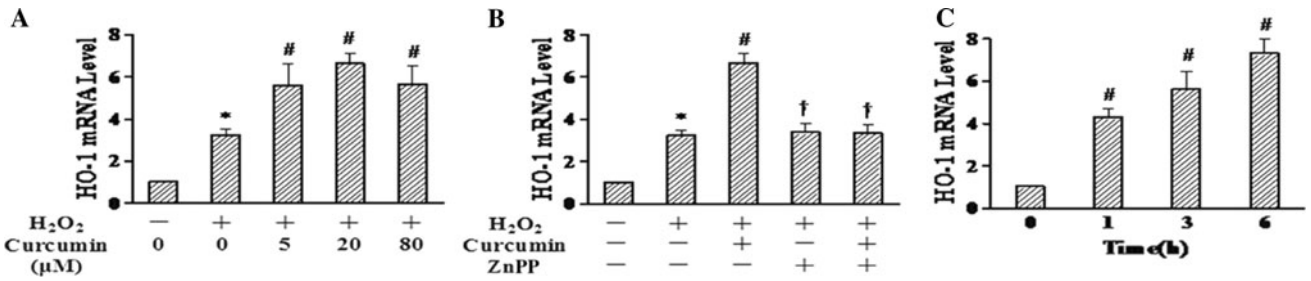

Fig. 4 Cur induced HO-1 mRNA expression in H_2O_2-induced Caco-2 cells. **a** Induction of HO-1 mRNA by Cur. Treatment Caco-2 cells with 500 μM H_2O_2 increased HO-1 mRNA expression, but pretreatment with 5, 20, and 80 μM Cur further potentiated HO-1 mRNA expression. **b** ZnPP blocked the induction of HO-1 mRNA by Cur. **c** Cur induced HO-1 mRNA expression at the times indicated. Expression of HO-1 mRNA was observed at 1 h and increased with time up to 6 h of pretreatment with Cur. HO-1 mRNA levels were determined by real time PCR. Values are presented as means ± SD ($^*P < 0.05$ compared with the control culture, $^{\#}P < 0.05$ compared with cells exposed to H_2O_2 alone, $^{\dagger}P < 0.05$ compared with cells treated with 20 μM Cur, $n = 3$)

GAPDH 115.00 ± 18.03, 175.67 ± 29.50%, 120.67 ± 32.72% vs 53.67 ± 24.19%, $P < 0.05$; ZO-1/GAPDH 90.33 ± 22.50, 139.67 ± 33.71%, 92.00 ± 24.33% vs 36.00 ± 15.88%, $P < 0.05$; Fig. 5a). Caco-2 cells were treated with Cur at 20 μM for 30 min before 500 μM H_2O_2 administration for the indicated time. However, incubation of Caco-2 monolayers with ZnPP blocked the protective effects of Cur on H_2O_2-induced decrease in occludin (occludin/GAPDH 54.67 ± 10.02, 168.33 ± 36.47%, $P < 0.05$) and ZO-1 expression (ZO-1/GAPDH 50.00 ± 15.13, 117.67 ± 38.81%, $P < 0.05$; Fig. 5b). Expression of occludin and ZO-1 were observed increased at 1 h and with time up to 6 h of Cur treatment (occludin/GAPDH 185.00 ± 51.64, 300.67 ± 27.10%, 424.33 ± 69.66% vs 84.00 ± 19.00%, $P < 0.05$; ZO-1/GAPDH 48.67 ± 20.01, 82.00 ± 12.12%, 114.67 ± 20.50% vs 15.00 ± 12.53%, $P < 0.05$; Fig 5c).

The localization of occludin and ZO-1 were observed by immunofluorescence microscopy. In the control cells, the expression of occludin was intact and high on the cell surface. H_2O_2 treatment for 6 h disrupted the integrity of occludin with lower expression on the cell surface. Pretreatment with 20 μM Cur significantly prevented the H_2O_2-induced disturbance of occluding, restoring the cell integrity and promoting expression of occludin. ZnPP could block the protective effect of Cur on H_2O_2-induced redistribution of occludin. Changes of ZO-1 were similar to those of occludin (Fig. 6).

Discussion

Throughout the gastrointestinal tract, a single layer of epithelial cells acts as a gateway that restricts uncontrolled entry of pathogens, toxins, and allergens. This function is critical for maintaining mucosal homeostasis, as is evident from colonic inflammation associated with loss of epithelial TJ integrity [1, 2]. The importance of maintaining the integrity of TJ has been emphasized by recent reports indicating that disruption of TJ and the consequent increase in paracellular permeability are of crucial importance in the pathogenesis of gastrointestinal disorders. The mechanisms involved in barrier dysfunction in different diseases are not well understood. However, a growing body of evidence indicates that a variety of reactive oxygen species including H_2O_2 disrupt epithelial barrier function in the gastrointestinal tract [10, 13, 14].

Hydrogen peroxide (H_2O_2) is the specific oxidant species responsible for cell injury in the Caco-2 cell monolayers model. Previous studies showed that H_2O_2, but not superoxide or hydroxyl radical, disrupted the TJ and increased paracellular permeability [10, 13, 14] by enhancing tyrosine phosphorylation of several proteins in Caco-2 cell monolayers [14]. Previous study has shown that treatment with 500 μM H_2O_2 causes a significant decrease in TEER in Caco-2 cell monolayers [13]. This is consistent with our study further showing that 500 μM H_2O_2 treatment reduced TEER in a time-dependent manner indicating that H_2O_2 disrupts mucosa barrier integrity in Caco-2 cell monolayers, which is also supported by the evidence that H_2O_2 treatment significant increased sodium fluorescent flux in Caco-2 cell monolayers. These results suggested that H_2O_2 induced a break in the mucosal barrier of the Caco-2 monolayer. Furthermore, cell monolayers treated with H_2O_2 for 6 h induced oxidative stress and cytotoxic effects, increasing LDH activity. This is consistent with a previous study showing that H_2O_2 treatment at 150 μM for 30 min increased LDH activity in Caco-2 cells [24]. However, another study indicated that H_2O_2 stimulation at 500 μM for 6 h did not change LDH activity at any time point in Caco-2 cells [14].

TJ are of crucial importance in creating an effective and stable barrier to maintain body homeostasis. The proteins spanning the cytoplasmic membrane, for example occludin and cytoplasmic proteins which link these membrane proteins to the cytoskeleton, for example ZO-1, could be distinguished in the constituting elements of TJ. Occludin is the most studied transmembrane protein localized in TJ. Previous studies have shown that occludin is of major

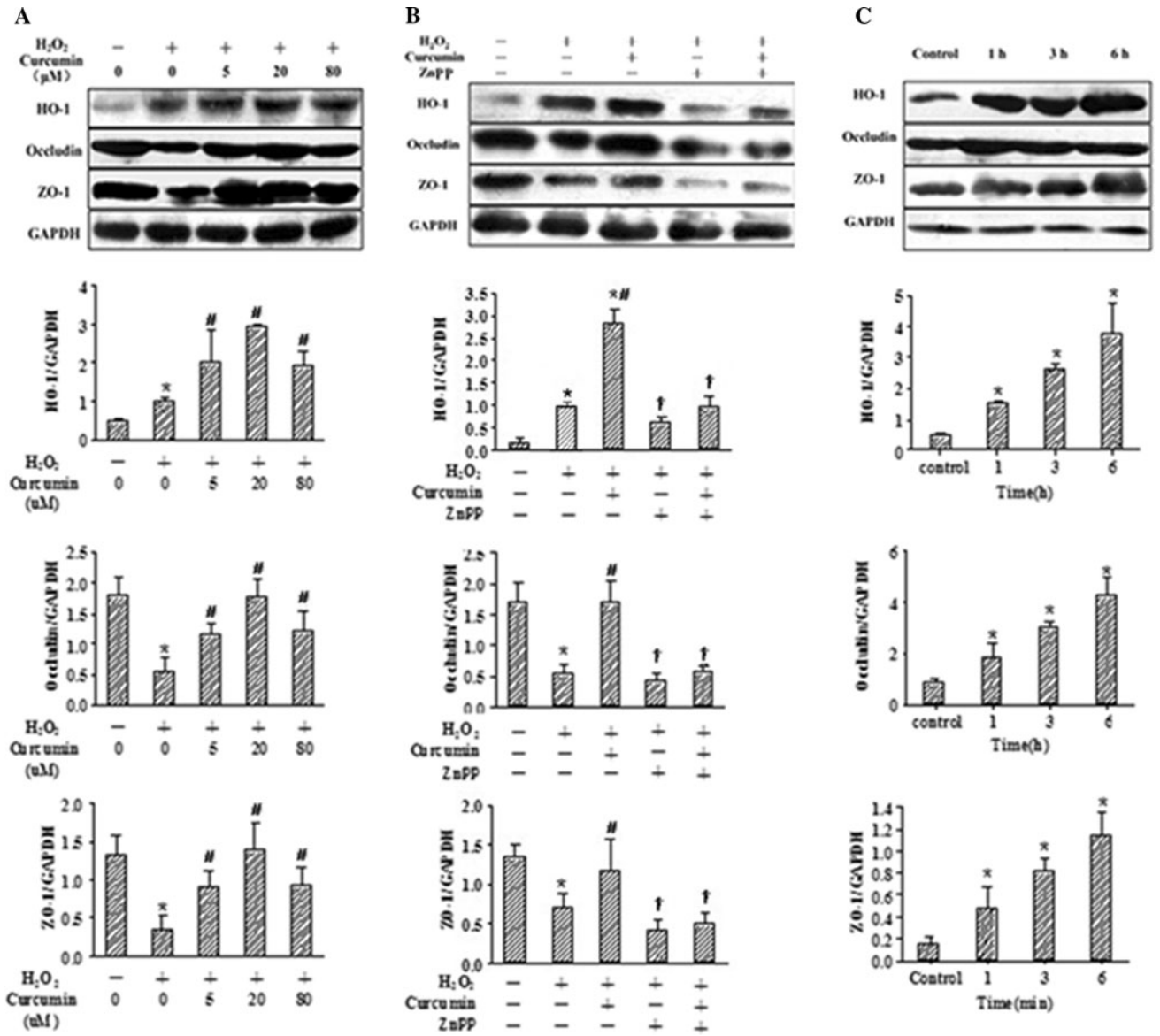

Fig. 5 Cur ameliorated H_2O_2-induced TJ disruption via upregulation HO-1 protein expression in Caco-2 cells (**a**) Cur induced HO-1 expression and protected H_2O_2-induced TJ disruption in Caco-2 cells in a partially concentration-dependent manner; 20 μM was the best concentration. (**b**) Cur ameliorated H_2O_2-induced TJ disruption via upregulation HO-1 expression in human intestinal epithelial cells. **c** Cur induced HO-1 expression and protected against H_2O_2-induced TJ disruption in time-course in Caco-2 cells. Values are presented as means ± SD ($^*P < 0.05$ compared with the control culture, $^{\#}P < 0.05$ compared with cells exposed to H_2O_2 alone, $^{\dagger}P < 0.05$ compared with cells treated with 20 μM Cur, $n = 3$)

importance in the regulation of TJ barrier function and is recruited by ZO-1 to cell junctions [3, 4, 25] Therefore, occludin and ZO-1 are crucial for the assembly of TJ and the epithelial barrier function. Indeed, numerous studies have shown that loss of occludin and ZO-1 protein or reassembly of these proteins could lead to a barrier dysfunction. Our results also showed that H_2O_2 reduced occludin and ZO-1 expression, interfering with TJ assembly, leading to disrupted Caco-2 cell integrity. Therefore, compounds that prevent H_2O_2-mediated disruption of the TJ by restoring occludin and ZO-1 expression may provide potential therapeutic benefit in the treatment of many gastrointestinal diseases.

The intestinal epithelial barrier-protective effects of Cur have been repeatedly demonstrated in a variety of gastrointestinal (GI) disease models [26–28]. However, the exact mechanisms underlying its intestinal epithelial barrier protective actions are not yet fully elucidated. It has previously been demonstrated that Cur is capable of inducing several endogenous antioxidants in cultured intestinal disorders and of reducing mucosal injury in trinitrobenzene sulfonic acid-induced colitis in vivo [27, 28]. LDH and MDA level are

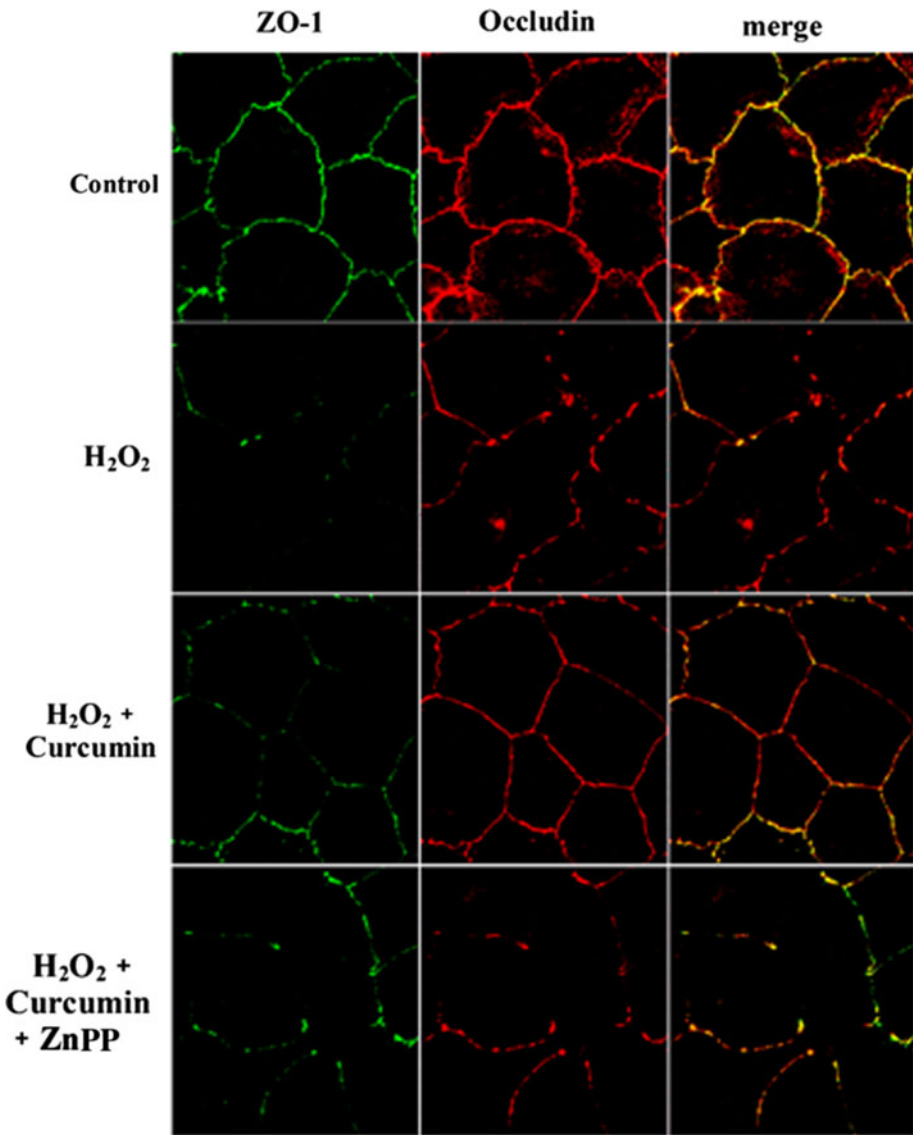

Fig. 6 Cur prevented H_2O_2-induced redistribution of TJ proteins via HO-1. Caco-2 cell monolayers were incubated with Cur before administration of H_2O_2 and 6 h after H_2O_2 administration. In some groups, cell monolayers were retreated with ZnPP at 20 μM for 12 h before administration of Cur. Cell monolayers were fixed in buffered 4% paraformaldehyde and labeled for occludin (*red*) and ZO-1 (*green*) by immunofluorescence staining. Images were collected by using a confocal laser-scanning microscope. Treatment of Caco-2 cell monolayers with 500 μM H_2O_2 resulted in loss of occludin and ZO-1. Pretreatment with Cur partially reversed the effect of H_2O_2-induced reduction, and ZnPP blocked the effect of Cur. In the control cells, the expression of occludin was normal and fluorescence intensity was high

associated with oxidative stress and SOD is believed to have anti-oxidant activity. Our study shows that Cur could attenuate H_2O_2-induced oxidative stress and the disrupted integrity in Caco-2 cells by reducing oxidative stress levels by increasing anti-oxidative effect.

HO-1 is a part of the cell's natural defense mechanisms. The HO-1 pathway seems to be of crucial importance in the preservation of tissue integrity against oxidative stress, and acts in synchrony with other crucial enzymatic systems in the maintenance of cellular homeostasis [29, 30]. Cur is a naturally occurring compound which is known to induce HO-1 production, although the underlying mechanism has not been fully elucidated. In recent years Cur has been shown to have cytoprotective properties by inducing the protective protein HO-1 [18, 21, 31]. Although previous study has shown that Cur induces HO-1 expression in hepatocytes and prevents ethanol-induced oxidative damage [27], the protective effect of Cur on intestinal diseases still needs to be elucidated. In this study, we demonstrated that Cur induced HO-1 expression in a time-dependent manner at both the mRNA and protein levels, which provides a possible mechanism of the action of Cur against the H_2O_2-induced increase in paracellular permeability in Caco-2 cells. The other possible protection mechanism is that Cur protects TJ integrity by regulation expression of TJ components. This hypothesis was supported by a study showing that Cur prevents TNF-α-induced decrease in ZO-1 protein levels in Caco-2 cell layers [28]. Our current study provided further evidence from different injury stimuli that Cur restores occludin and ZO-1 protein levels after H_2O_2 treatment. On the other hand, ZnPP not only exerts cellular effects independent of HO-1 activity but is also widely used as an HO-1 inhibitor in several model systems [32]. In addition, our current

study further supported the fact that ZnPP, a commonly used specific HO-1 inhibitor, can block the protection from Cur, which indicates Cur protects Caco-2 cell integrity by inducing HO-1 expression. The attenuation of Cur-mediated protection of H_2O_2-induced disruption by ZnPP clearly demonstrates that HO-1 acts by intestinal epithelial protection.

In conclusion, despite the fact that the Cur exerts its protection of epithelial dysfunction through multiple mechanisms, this in-vitro study shows that Cur is a potent inducer of HO-1 and can ameliorate the oxidative stress-induced disruption of intestinal epithelial TJ by an HO-1-dependent mechanism. It also provides a rationale to develop Cur preparations for treatment of intestinal disorder.

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Conflict of interest None.

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