Amelioration of hypoxia and LPS-induced intestinal epithelial barrier dysfunction by emodin through the suppression of the NF-κB and HIF-1α signaling pathways

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Abstract. Intestinal barrier dysfunction occurs in critical illnesses and involves the inflammatory and hypoxic injury of intestinal epithelial cells. Researchers are still defining the underlying mechanisms and evaluating therapeutic strategies for restoring intestinal barrier function. The anti-inflammatory drug, emodin, has been shown to exert a protective effect on intestinal barrier function; however, its mechanisms of action remain unknown. In this study, we investigated the protective effects of emodin on intestinal barrier function and the underlying mechanisms in intestinal epithelial cells challenged with lipopolysaccharide (LPS) and hypoxia/reoxygenation (HR). To induce barrier dysfunction, Caco-2 monolayers were subjected to HR with or without LPS treatment. Transepithelial electrical resistance and paracellular permeability were measured to evaluate barrier function. The expression of the tight junction (TJ) proteins, zonula occludens (ZO)-1, occludin, and claudin-1, as well as that of hypoxia-inducible factor (HIF)-1α, phospho-IκB-α, phospho-nuclear factor (NF)-κB p65 and cyclooxygenase (COX)-2 was determined by western blot analysis. The results revealed that emodin markedly attenuated the decrease in transepithelial electrical resistance and the increase in paracellular permeability in the Caco-2 monolayers treated with LPS and subjected to HR. Emodin also markedly alleviated the damage caused by LPS and HR (manifested by a decrease in the expression of the TJ protein, ZO-1), and inhibited the expression of HIF-1α, IκB-α, NF-κB and COX-2 in a dose-dependent manner. In conclusion, our data suggest that emodin attenuates LPS- and HR-induced intestinal epithelial barrier dysfunction by inhibiting the HIF-1α and NF-κB signaling pathways and preventing the damage caused to the TJ barrier (shown by the decrease in the expression of ZO-1).

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Introduction

A number of critical illnesses, such as shock, trauma and burns, as well as cardiac and abdominal surgery and small intestinal transplantation, can lead to intestinal ischemia/reperfusion (I/R) injury, which destroys intestinal barrier function (1). The loss of this function is a key event in the development of gut-derived sepsis, which perpetuates or worsens critical illness. Sepsis is a major healthcare issue, affecting millions of individuals worldwide each year (2-4). The gut is considered the 'motor' of sepsis and multiple organ dysfunction syndrome (MODS); therefore, it is of critical importance to establish optimal therapeutic strategies to protect intestinal barrier function (5).

The hypoxia-inducible factor (HIF)-1 heterodimer consists of oxygen-labile HIF-1α and constitutively expressed HIF-1β. The transcription factor, HIF-1, mediates a wide spectrum of physiological and cellular adaptive responses, such as angiogenesis, metabolic adaption, erythropoiesis and vascular tone (6-9). Apart from hypoxia, cytokines (10) and bacteria (11,12) are also capable of activating HIF-1α in enterocytes. A number of recent studies have suggested that HIFs play an important role in intestinal barrier function (13-20); however, the exact molecular mechanisms involved are not yet fully understood. In certain studies, HIF responses have been shown to be a part of disease recovery, whereas others have proven that the responses promote disease progression. In the studies by Kelly *et al*, Clambey *et al*, Kong *et al*, Keely *et al* and Lee *et al*, it was demonstrated that HIF-1α is a protective factor in intestinal inflammation, restoring intestinal integrity and epithelial innate immunity (13-17). However, Liu *et al* confirmed that the inhibition of HIF-1α attenuates intestinal epithelial barrier dysfunction (18). Kannan *et al* and Feinman *et al* proved that HIF-1 plays a gut-injurious role in intestinal injury (1,19). Yang *et al* reported that interferon (IFN)-γ induces epithelial barrier dysfunction and the disruption of tight junctions (TJs) by upregulating HIF-1α expression through the nuclear factor (NF)-κB pathway (20). Peyssonnaux *et al* demonstrated that the inhibition of HIF-1α activity may represent a novel

therapeutic target for lipopolysaccharide (LPS)-induced sepsis (21,22). Taken together, the complex roles of HIF-1α and the different activated signaling pathways in the intestinal barrier remain to be fully elucidated.

NF-κB is an important regulator of inflammatory signaling, containing p65 (RelA), RelB, c-Rel, p50 (NF-κB1) and p52 (NF-κB2). There are two primary activation pathways for NF-κB. The canonical signaling pathway is dependent on IKK-β activation. LPS, TNF-α or IL-1 activate each respective receptor. This leads to an activation of IKK-β in the IKK complex, which can then phosphorylate IκB-α, which in turn results in the degradation of IκB-α. NF-κB can then translocate to the nucleus. The non-canonical signaling pathway is dependent on IKK-α activation. Through a variety of adapter proteins and signaling kinases, this leads to the translocation of NF-κB (23). Post-translational modifications, including acetylation and phosphorylation are vital to NF-κB transcriptional activity (24).

The NF-κB and HIF pathways are intimately associated and there is a significant level of crosstalk between these pathways at a number of levels (25,26). However, the relative contribution of each to hypoxia or inflammation remains unclear. In the inflammatory response, cyclooxygenase (COX)-2 is a key enzyme in gut barrier failure during murine peritonitis (27). Fitzpatrick *et al* reported that COX-2, which is under the control of HIF-1, is also dependent on the presence of an intact canonical NF-κB signaling pathway (28). COX-2 contains functional response elements for both HIF-1 and NF-κB; however, the relative contribution of these transcriptional regulators to hypoxia and inflammation remains controversial (29-31).

Emodin is a natural anthraquinone compound that is isolated from the traditional Chinese medicine, *Rheum palmatum* (32). It has been shown to possess antibacterial, antitumor, anti-inflammatory and vasorelaxant effects (33-35). *Rheum palmatum* has long been used for acute intestinal obstruction in China. As a laxative, it has been proven to regulate the contractility of intestinal smooth muscle (36). However, the mechanisms underlying the effects of emodin on intestinal barrier function are not yet completely understood.

In the present study, we hypothesized that emodin modulates intestinal barrier injury which is induced by LPS and hypoxia/reoxygenation (HR) in intestinal cells *in vitro*. We demonstrate that emodin significantly attenuates the increase in paracellular fluorescein isothiocyanate (FITC)-dextran flux and inhibits the decrease in transepithelial electrical resistance (TEER) caused by LPS and HR. We assessed that emodin attenuated the LPS- and HR-induced intestinal epithelial barrier dysfunction through the inhibition of COX-2, mediated by HIF-1α and NF-κB. In addition, emodin attenuated the disruption of TJ barrier function, determined by the expression of zonula occludens (ZO)-1.

Materials and methods

Cell culture. Caco-2 cells (a human colonic cell line) obtained from the American Type Culture Collection (Manassas, VA, USA) were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 15% fetal bovine serum, 4.0 mM l-glutamine, 1% non-essential amino acids, 100 U/ml penicillin and 100 U/ml streptomycin (all from Invitrogen Life Technologies, Carlsbad, CA, USA). The cells were maintained in a humidified 37°C, 5% CO_2 incubator, and passaged by partial

digestion with 0.25% trypsin and 0.53 mM EDTA (Biochrom AG, Berlin, Germany) in Hank's balanced salt solution (HBSS; Invitrogen Life Technologies) without Ca^{2+} and Mg^{2+}.

MTT assay. Cytotoxic properties were assessed by MTT assay. The Caco-2 cells were seeded at a density of 10^5 cells/well in 96-well plates for 24 h to promote attachment. The medium was replaced with fresh medium containing various concentrations of emodin (Tianjin Institute for Drug Control, Tianjin, China; 20, 40, 60 or 80 μM) for 72 h. Following treatment, the cells were incubated in the dark with MTT solution (Invitrogen Life Technologies) for 4 h at 37°C. The solution was aspirated and the formazan crystal product was solubilized in 100 μl DMSO. The absorbance was measured at 570 nm using a microplate ELISA reader. All experiments were carried out in triplicate and the results are presented as the means ± SEM.

Monolayer preparation and treatment. The cells were plated at a density of $5x10^4/cm^2$ on collagen-coated permeable polycarbonate membrane Transwell supports with 0.4 μm pores (Corning, Corning, NY, USA) and were grown as monolayers prior to the experiments. In the normoxia control experiments, the cells were cultured at 37°C in a humidified incubator containing 95% air and 5% CO_2, using pre-equilibrated normoxic medium. In the LPS experiments, the cell monolayers were treated with various concentrations of LPS from *Escherichia coli* 111:B4 (10^{-6}-1 mg/ml; Sigma, St. Louis, MO, USA) for 0.5-24 h. In the hypoxia experiments, the cells were placed in a sealed modular incubator chamber-101 (Billups-Rothenberg, Inc., Del Mar, CA, USA), which was packed with gas consisting of 94% N_2, 5% CO_2 and 1% O_2 for 1-4 h, using pre-equilibrated hypoxic medium. The oxygen concentration was measured using the oxygen detector, SPD201/O_2 (Shenzhen Sanpo Instrument Co., Ltd., Shenzhen, China). In the reoxygenation experiments, the cells were moved out of the hypoxia chamber after 3 h of exposure to hypoxia, and then maintained under normoxic conditions for 1-4 h with the replacement of fresh pre-equilibrated normoxic medium. In the emodin experiments, LPS and various concentrations of emodin (Tianjin Institute for Drug Control) were simultaneously added to the apical chamber.

Measurement of TEER. TEER values, an indicator of TJ permeability to ionic solutes, were measured using a Millicell-ERS voltohmmeter (Millipore, Bedford, MA, USA). Each measurement was calculated by subtracting the resistance value of the filters and fluids, and expressed as a percentage of the initial values.

Intestinal paracellular permeability assay. To investigate intestinal permeability, we used the flux of FITC-conjugated dextran (molecular weight, 4 kDa) (Sigma) as a probe. The movement of FITC-dextran across the monolayers represented the apical-to-basal paracellular permeability of the intestinal barrier. The cells were treated with LPS (1 μg/ml), $H_{3h}R_{3h}$ + LPS or $H_{3h}R_{3h}$ + LPS + emodin (60 μmol/l) for 6 h. The apical chamber was filled with 100 μl of the different solutions with 1 mg/ml FITC-dextran in HBSS and the basolateral chamber was filled with 600 μl of growth medium followed by incubation at 37°C. Basolateral samples (100 μl) were taken every 1 h over the last 3 h, replenishing with fresh medium at each sample time point.

Fluorescence was determined using a fluorescence microplate plate reader (Fluorescence Spectrophotometer F-7000; Hitachi High Technologies America, Inc., Schaumburg, IL, USA). Apparent permeability co-efficients (P_{app}) were then calculated according to the following equation:

$$P_{app} = \left(\frac{dQ}{dt} \right) \times \left(\frac{1}{A \times C_0} \right)$$

where dQ/dt is the transport rate (mg/sec), A is the cell mono-layer surface area (cm^2) and C_0 is the initial concentration in the donor compartment (mg/ml).

Western blot analysis. The Caco-2 monolayers were washed twice with ice-cold PBS, and then lysed in RIPA buffer with a cocktail of protease inhibitors on ice. Cell debris was separated by centrifugation at 15,000 x g for 20 min at 4˚C. The quantity of protein in the supernatants was deter-mined using a BCA protein assay kit (BioTeke Corporation, Beijing, China). Equal amounts of protein samples were separated by SDS-PAGE and were transferred onto PVDF membranes (Millipore). After blocking with 5% non-fat milk for 1 h, the membranes were incubated with primary antibodies specific for ZO-1 (Cat. no. 61-7300, dilution 1:1,000), occludin (Cat. no. 33-1500, dilution 1:1,000), claudin-1 (Cat. no. 37-4900, dilution 1:1,000; all from Invitrogen Life Technologies), HIF-1α (Cat. no. 610959, dilution 1:1,000; BD Biosciences, Franklin Lakes, NJ, USA), COX-2 (Cat. no. 35-8200, dilution 1:1,000; Invitrogen Life Technologies), phospho-NF-κB p65 (Ser536) (Cat. no. 3033, dilution 1:1,000), phospho-IκB-α (Ser32) (Cat. no. 2859, dilu-tion 1:1,000) and β-tubulin (Cat. no. 2128, dilution 1:1,000; all from Cell Signaling Technology, Inc., Danvers, MA, USA) overnight at 4˚C. After 3 washes, the membranes were incubated with peroxidase-conjugated secondary antibodies for 1 h. Proteins were detected using the enhanced chemilu-minescence detection kit (Millipore). Band intensities were quantified using Quantity One software (Bio-Rad, Hercules, CA, USA). Relative expression was normalized to β-tubulin.

Statistical analysis. The differences among multiple groups were assessed by one-way ANOVA using SPSS software version 13.0. All data are presented as the means ± SEM. All reported significance levels represent two-tailed P-values. A value of P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of emodin on cell viability. Emodin (<80 μM) did not exert any growth inhibitory or general cytotoxic effects on the Caco-2 cells (Fig. 1B). Emodin (0-80 μM) did not affect the viability of the intestinal epithelial cells *in vitro*.

Emodin attenuates intestinal barrier dysfunction induced by LPS and HR injury. It has been demonstrated that LPS or hypoxia causes intestinal barrier dysfunction (37-39). To investigate the effects of emodin on intestinal barrier func-tion, we used an *in vitro* model in which Caco-2 epithelial
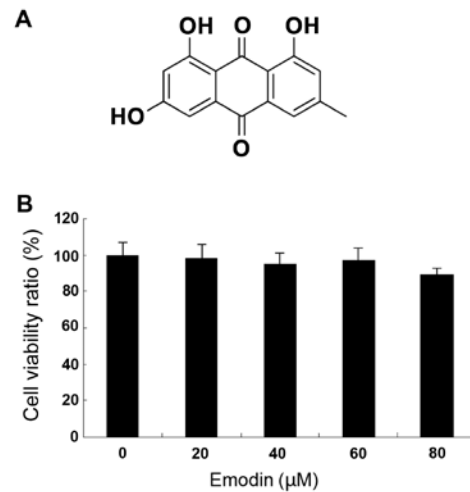

Figure 1. Effects of emodin on cell viability. (A) Structure of emodin. (B) Cells were treated with various concentrations of emodin for 6 h, prior to cytotox-icity being measured.

cell monolayers were treated with LPS and subjected to HR. TEER is an indicator of epithelial paracellular permeability to ionic solutes and was used to assess intestinal barrier function. Compared with the controls (untreated cells), TEER decreased following treatment with LPS or LPS with exposure to HR for 6 h, indicating the disruption of the barrier function in the monolayers (Fig. 2A). However, emodin significantly inhibited (P<0.05) the reduction in TEER induced by treatment with LPS and exposure to HR.

Consistent with the changes in TEER, P_{app} for FITC-dextran flux, an indicator of epithelial paracellular permeability to uncharged macromolecules, markedly increased when the Caco-2 monolayers were treated with LPS and exposed to $H_{3h}R_{3h}$ for 6 h (Fig. 2B). This indicated that the paracellular permeability to non-ionic macromolecules was increased by treatment with LPS and exposure to HR. As shown in Fig. 2B, treatment with emodin significantly reduced (P<0.05) the increase in the paracellular FITC-dextran flux induced by LPS and HR. These data suggest that treatment with emodin attenuates intestinal epithelial barrier dysfunction induced by LPS and HR *in vitro*.

Emodin prevents the disruption of TJ barrier function (shown by ZO-1) induced by LPS and HR injury. It has been demonstrated that alterations in the expression of TJ proteins are involved in intestinal barrier disruption induced by pro-inflammatory cytokines or burn injury (10,40). Thus, we examined the total expression of ZO-1, claudin-1 and occludin in Caco-2 monolayers treated with LPS, LPS + HR, and LPS + HR + emodin. The expression of occludin and claudin-1 was not significantly affected by treatment with LPS and/or hypoxia in the absence or presence of emodin (Fig. 3B and C). However, as shown in Fig. 3A, the expression of ZO-1 decreased significantly when the cells were treated with LPS and exposed to hypoxia, but emodin attenuated this reduction. This indicates that alterations in the expression of ZO-1 may be one of the mechanisms through which emodin protects against intestinal epithelial barrier dysfunction induced by exposure to hypoxia and treatment with LPS.
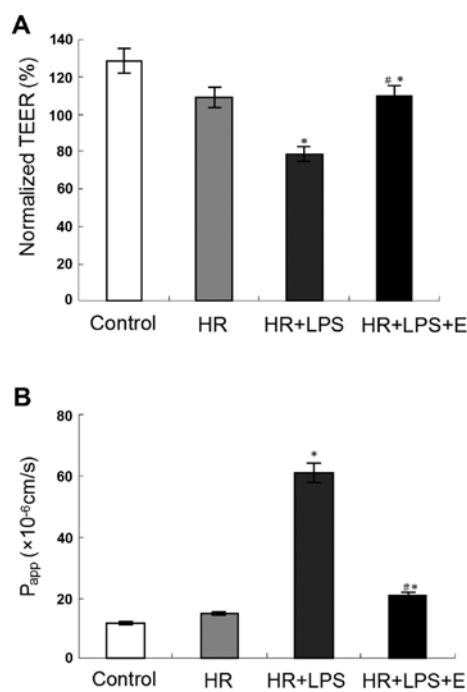
Figure 2. Emodin attenuates barrier dysfunction induced by hypoxia/reoxygenation (HR) and lipopolysaccharide (LPS) in Caco-2 monolayers. (A) Caco-2 monolayers were exposed to $H_{3h}R_{3h}$ and LPS (LPS 1 μg/ml, 1% O_2) and emodin (E; 60 μmol/l) was added to the basal chamber for 6 h. The designated conditions were control (medium only), $H_{3h}R_{3h}$ + LPS, $H_{3h}R_{3h}$ + LPS + emodin. Emodin significantly inhibited the decrease in transepithelial electrical resistance (TEER) induced by simultaneous exposure to HR and LPS. (B) Caco-2 monolayers were treated as described for (A). Apparent permeability co-efficients (P_{app}) of fluorescein isothiocyanate (FITC)-dextran flux. Data represent the means ± SEM. HR and LPS increased paracellular permeability to 4 kDa FITC-dextran. This increase was significantly decreased by treatment with emodin (n=5). $^*P<0.05$, compared with the controls. $^\#P<0.05$, compared with HR + LPS.

LPS induces the expression of HIF-1α and the hypoxia-responsive gene, COX-2, in a time- and dose-dependent manner. Different types of non-hypoxic cell stimulation have been shown to increase HIF-1α expression in macrophages (41,42); therefore, we wished to examine the effects of LPS on the expression of HIF-1α and the hypoxia-responsive gene, COX-2, in Caco-2 cells. The Caco-2 cells were stimulated with 10^{-6}-1 mg/ml LPS for 6 h, followed by the evaluation of HIF-1α protein levels by western blot analysis. The induction of HIF-1α expression by LPS was dose-dependent; an increase was observed at 10^{-6} mg/ml; maximal induction was observed at 10^{-3} mg/ml and a decrease was observed when the cells were treated with high doses of LPS (10^{-2}-1 mg/ml) (Fig. 4A and B). The trend in the expression of the downstream target gene, COX-2, was similar to that of HIF-1α (Fig. 4A and C).

In time course experiments, the cells were stimulated with 10^{-3} mg/ml LPS for different periods of time. The maximal induction of HIF-1α was attained after 30 min in the presence of LPS; after 30 min, HIF-1α levels decreased, and decreased to the minimum levels at 4 h; after 4 h HIF-1α expression increased again (Fig. 4D and E). The trend in the expression of COX-2 was generally similar to that of HIF-1α, although, COX-2 expression first showed a slight increase and then decreased to minimum levels at 4 h (Fig. 4D and F). These
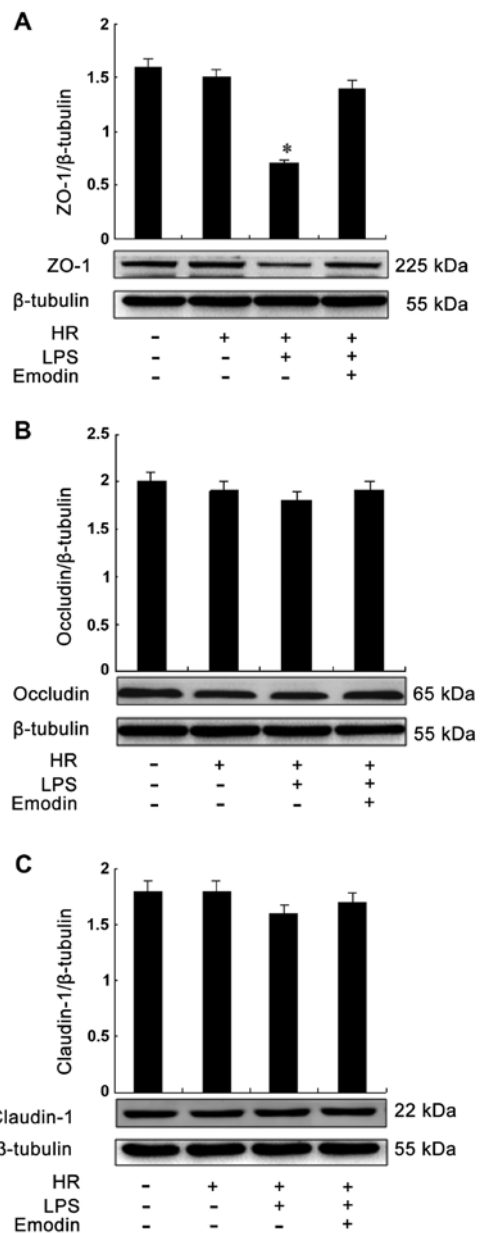

Figure 3. Emodin prevents the decrease in the expression of zonula occludens (ZO)-1 induced by lipopolysaccharide (LPS) and hypoxia/reoxygenation (HR) injury. Caco-2 monolayers were treated as described in Fig. 1A. Cell lysates were analyzed to detect the expression of (A) ZO-1, (B) occludin and (C) claudin-1 by western blot analysis. Only ZO-1 expression decreased significantly when the cells were exposed to LPS + hypoxia. Emodin attenuated the decrease in ZO-1 expression induced by LPS and HR injury (n=5). $^*P<0.05$, compared with controls.

results demonstrate that the stimulation of Caco-2 cells with LPS induces the expresionof HIF-1α and that of its downstream target gene ,COX-2, in a time- and dose-dependent manner.

The HIF-1α and NF-κB signaling pathways are activated by hypoxia and hypoxia + LPS. Both HIF-1α and NF-κB are the key oxygen-sensitive transcriptional regulators in inflammatory and hypoxic conditions. A recent study revealed a high degree of interdependence between the HIF and NF-κB signaling pathways, as well as a correlation between inflammation and hypoxia (28). However, the relative contribution of each transcriptional regulator to hypoxia and inflamma-
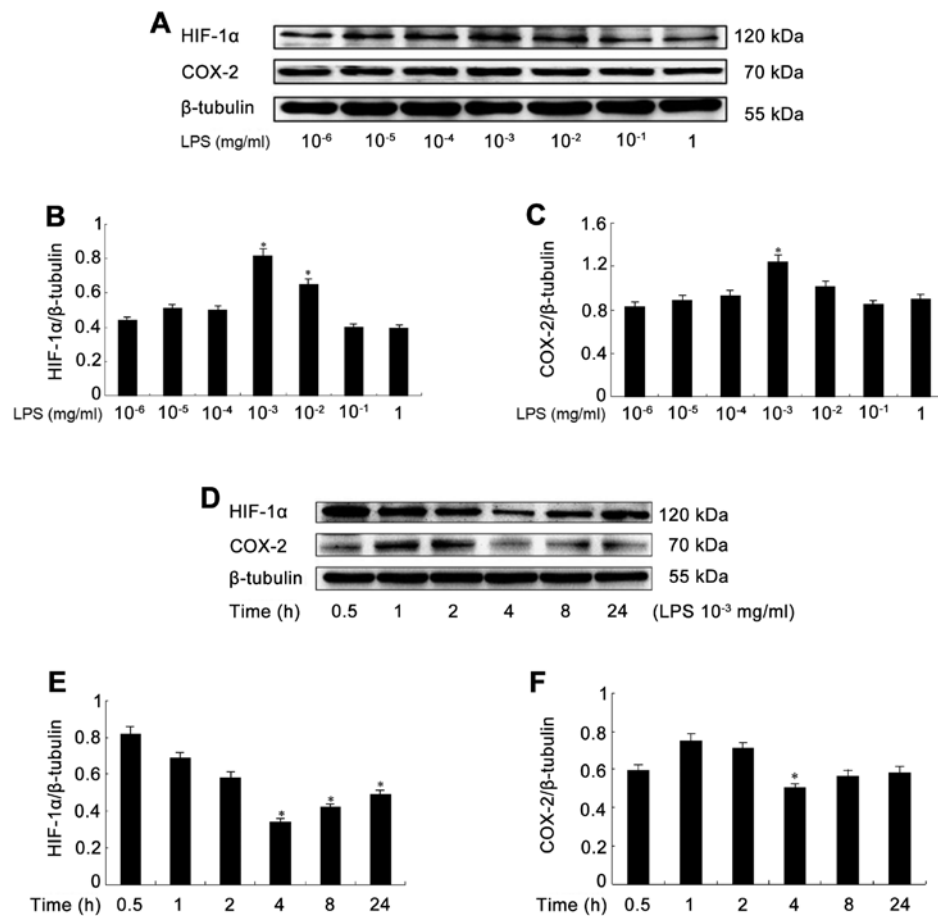
Figure 4. Treatment with lipopolysaccharide (LPS) induced hypoxia-inducible factor (HIF)-1α and cyclooxygenase (COX)-2 expression in Caco-2 cells in a dose- and time-dependent manner. (A-C) The cells were treated with LPS at different doses (10^{-6}-1 mg/ml) for 6 h. $^{*}P<0.05$, compared with 10^{-6} mg/ml group. (D-F) The cells were treated with LPS (10^{-3} mg/ml) for different periods of time (0.5-24 h). $^{*}P<0.05$, compared with 0.5 h group.

tion remains unclear. Thus, we determined the changes in the HIF-1α and NF-κB signaling pathways in Caco-2 cells exposed to hypoxia and hypoxia + LPS. Under hypoxic conditions alone, the expression of IκB, NF-κB and COX-2 showed a similar trend, decreasing as the duration of hypoxia increased (Fig. 5A, C-E). The change in HIF-1α expression differed, showing a slight increase and then a decrease (Fig. 5A and B). In the cells exposed to hypoxia + LPS, the trend for the expression of IκB, NF-κB, COX-2 and HIF-1α was similar. There was an increase at $H_{1-2\,h}$; maximal induction was observed at $H_{3\,h}$; a decrease was observed thereafter (Fig. 5).

The HIF-1α and NF-κB signaling pathways are activated by HR and HR + LPS. In the cells exposed to HR, the expression of IκB, NF-κB and COX-2 showed a similar trend, first increasing and then decreasing as the duration of reoxygenation increased (Fig. 6A, C-E). In the cells exposed to HR, the expression of HIF-1α decreased with reoxygenation, and the minimum induction was observed at $H_{3\,h}R_{1-2\,h}$ (Fig. 6A and B). In the cells exposed to HR + LPS, the trend in the expression of all 4 proteins was similar. An increase was observed at $H_{1-2\,h}$; maximal induction was attained at $H_{2-3\,h}$; a decrease was observed thereafter (Fig. 6).

Emodin inhibits the activation of the HIF-1α and NF-κB signaling pathways. Having demonstrated the protective effects

of emodin on intestinal barrier function *in vitro*, we then sought to determine whether emodin attenuates LPS + HR-induced barrier dysfunction by inhibiting the HIF-1α and NF-κB signaling pathways. The LPS + HR-induced acvitation of the HIF-1α and NF-κB pathways was significantly inhibited by emodin in a dose-dependent manner (Fig. 7A, B and D). This suggests that emodin protects intestinal barrier function against LPS + HR-induced injury by blocking the activation of the HIF-1α and NF-κB signaling pathways. Following treatment with emodidn, the expression of IκB and COX-2 showed a similar trend (Fig. 7A, C and E).

Discussion

It is well known that the gut is the 'motor' of critical illness and the origin of sepsis in a number of intensive care patients. The balance between the intestinal epithelium, immune system and endogenous microflora of the gut breaks down, leading to the development of gut-origin systemic diseases (43). A feed-forward loop possibly exists between intestinal barrier dysfunction and systemic injury. Unless broken, a continuous cycle of injury can lead to serious consequences. Treatment strategies for sepsis and other critical illness should focus on protecting the gut from inflammation and HR injury.

It has been demonstrated that LPS or HR disrupts intestinal epithelial barrier function both *in vitro* and *in vivo* (37-39).
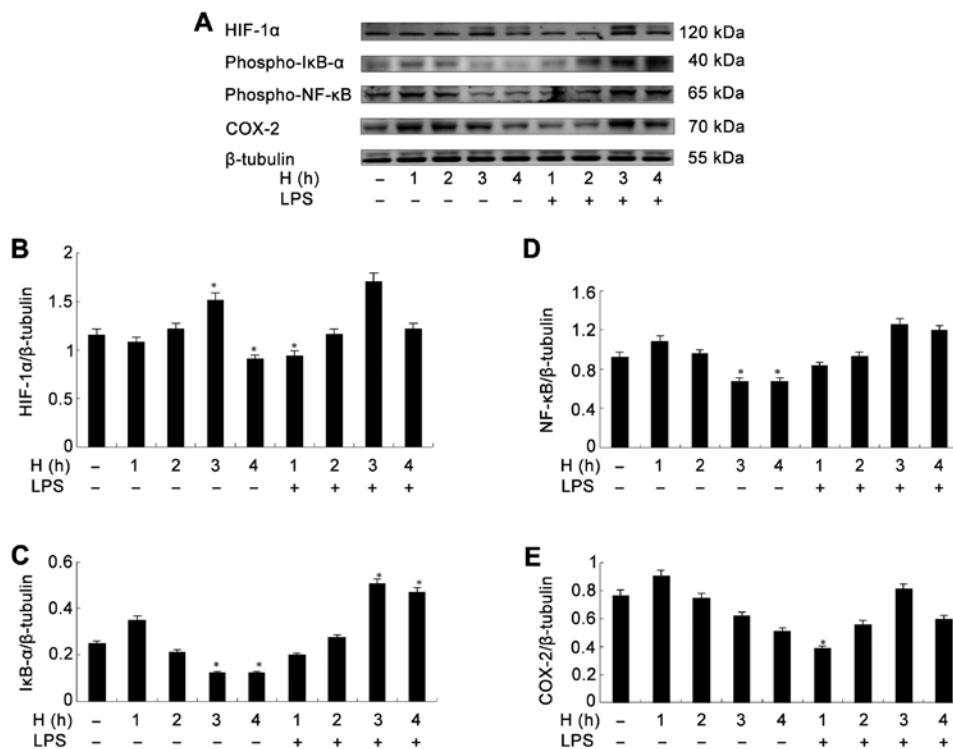
Figure 5. Exposure to hypoxia (H) or hypoxia + lipopolysaccharide (LPS) activated the hypoxia-inducible factor (HIF)-1α and nuclear factor (NF)-κB signaling pathways in Caco-2 cells. (A) Western blot analysis of protein expression. (B-E) In the cells exposed to hypoxia, the expression of IκB, NF-κB and COX-2 showed similar trends, which decreased as the duration of hypoxia increased, and the minimum induction was observed at $H_{3-4\,h}$. The change in HIF-1α expression was a small increase and a decrease thereafter. The maximal induction of HIF-1α expression was at $H_{3\,h}$. In the cells exposed to hypoxia + LPS, the trends for IκB, NF-κB, COX-2 and HIF-1α were similar. There was an increase at $H_{1\,h}$; maximal induction was attained at $H_{3\,h}$; and there was a decrease thereafter. *$P<0.05$, compared with controls.
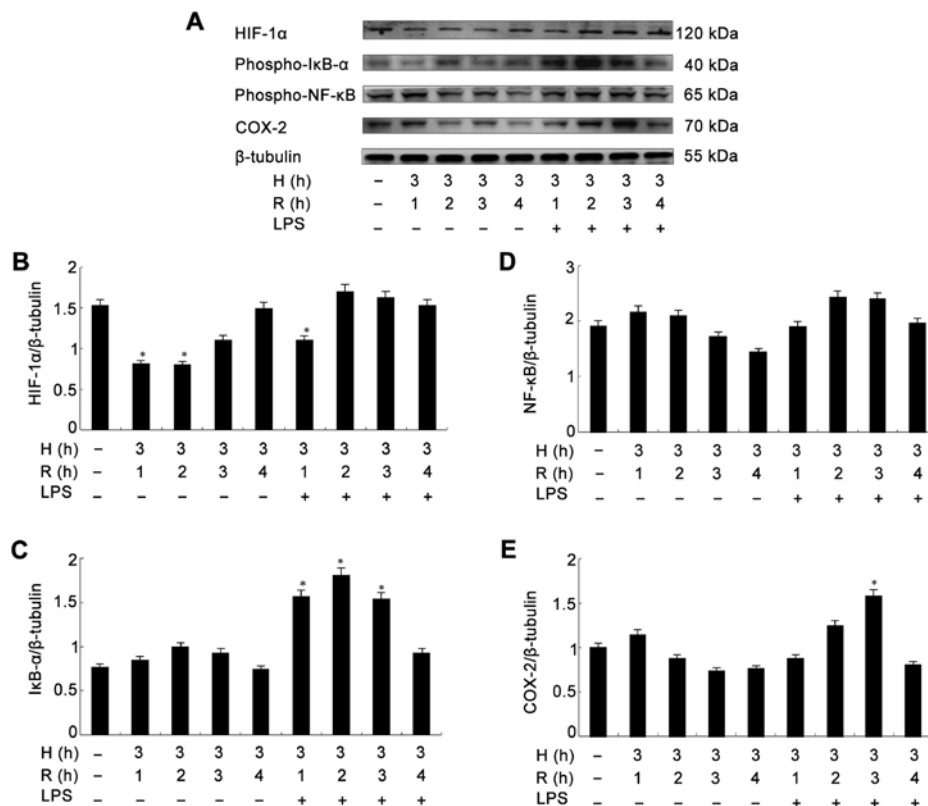

Figure 6. Hypoxia (H)/reoxygenation (R) (HR) or HR + lipopolysaccharide (LPS) activated the hypoxia-inducible factor (HIF)-1α and nuclear factor (NF)-κB signaling pathways. (A) Western blot analysis of protein expression. (B-E) In the cells exposed to HR, the expression of IκB, NF-κB and COX-2 showed a similar trend, an increase first and then a decrease with reoxygenation. The expression of HIF-1α decreased with reoxygenation, and the minimum induction was observed at $H_{3\,h}R_{1-2\,h}$. In the cells exposed to HR + LPS, the trends for all 4 proteins were similar. An increase was observed at $H_{1\,h}$; maximal induction was attained at $H_{3\,h}R_{2-3\,h}$; and a decrease was observed thereafter. *$P<0.05$, compared with controls.
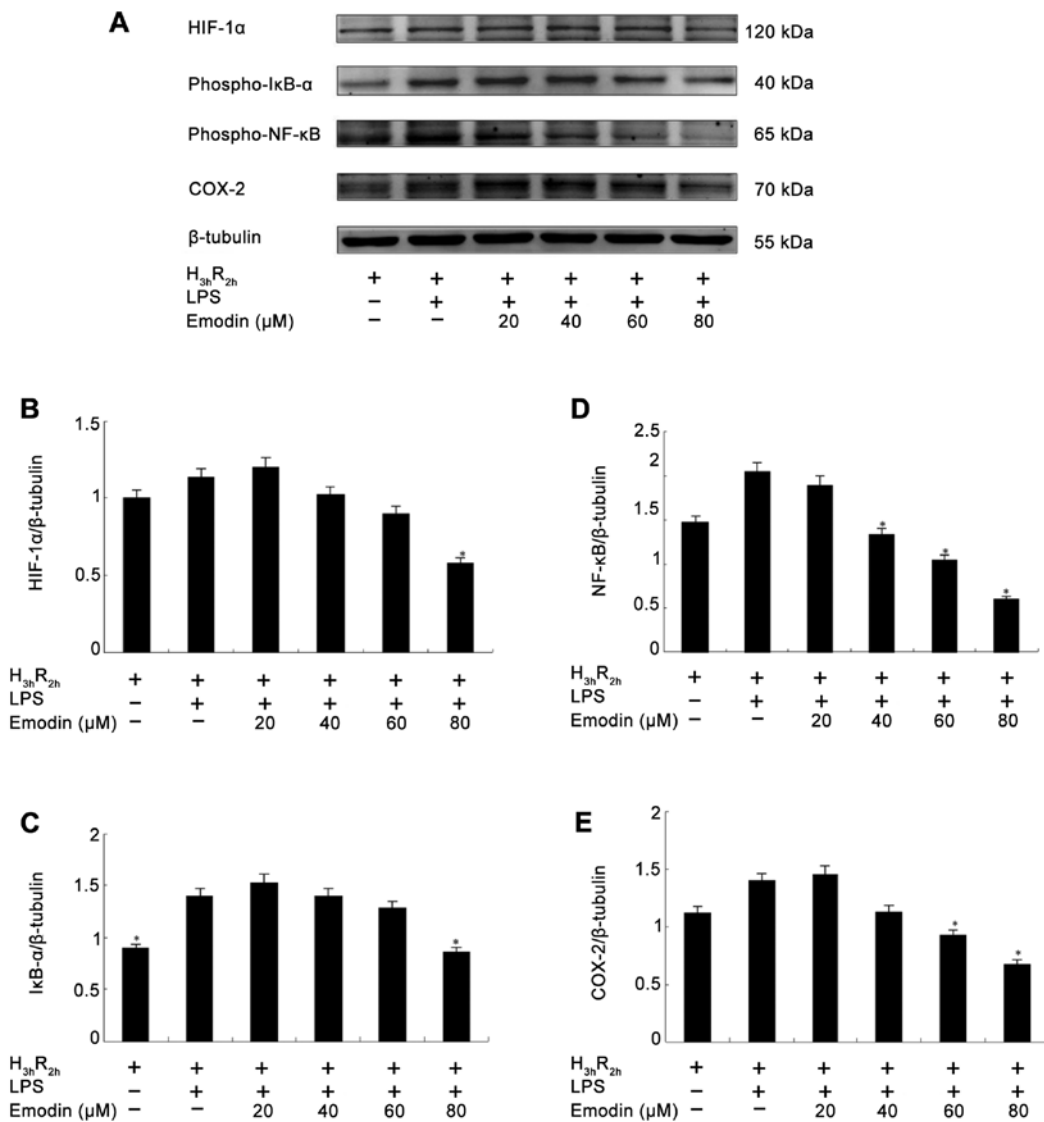
Figure 7. (A-E) Effects of emodin on the expression of hypoxia-inducible factor (HIF)-1α, cyclooxygenase (COX)-2, phospho-IκB-α and phospho-nuclear factor (NF)-κB p65 induced by exposure to lipopolysaccharide (LPS) and hypoxia/reoxygenation (HR). The cells were treated with LPS + $H_{3h}R_{2h}$ and different concentrations of emodin. Emodin inhibited the HIF-1 and NF-κB signaling pathways in a dose-dependent manner. The minimum induction of protein expression was observed following treatment with emodin at the concentration of 80 μmol/l. However, emodin is insoluble in water which is the main components of the medium. Emodin will be not soluble if too much is added to medium; 80 μM of emodin is already a rather high concentration. We tried 100 μM, the crystal was observed at the bottom of the culture flask. *$P<0.05$, compared with LPS + $H_{3h}R_{2h}$.

However, the molecular mechanisms involved have not yet been fully elucidated. In this study, we demonstrated that LPS and HR caused intestinal epithelial barrier dysfunction by decreasing TEER, increasing paracellular permeability and breaking the TJ barrier (shown by decrease in ZO-1 expression) in vitro (Fig. 8).

The intestinal epithelial barrier plays a significant role in preventing antigens and pathogens from entering the intestinal mucosa and encountering the immune system (44-46). TJs seal the paracellular space between epithelial cells and regulate the movement of fluid and macromolecules between the bloodstream and the intestinal lumen, and are critical to the maintenance of the integrity of the intestinal epithelial barrier (47). They are protein structures that represent the intestinal paracellular pathway of the intestinal barrier and are regarded as a key factor in intestinal permeability (48). In our study, LPS and HR damaged the TJ barrier (decreasing ZO-1 expression), leading to increased permeability of the intestinal barrier. This mechanism may, at least in part, contribute to the feed-forward loop between intestinal barrier dysfunction and critical illness. Thus, the protection of TJs may be a worthwhile target of therapeutic strategies for gut-derived sepsis.

Under normal oxygen conditions, HIF-α is rapidly destroyed through the proteasomal degradation pathway in the cytoplasm. By contrast, hypoxia or inflammation is associated with the stabilization of HIF-α. When HIF-α is stabilized, it can translocate to the nucleus and form a heterodimer with the HIF-1β subunit, allowing for transcriptional activity. HIF-α has 3 isoforms, HIF-1α, HIF-2α and HIF-3α. HIF-1α and HIF-2α have common and distinguishing characteristics in hypoxia and inflammation (49,50). The role of HIFs in the intestinal barrier function remains controversial. Studies have investigated the association of HIF-1α and intestinal barrier function (13-20); however, this association is not yet fully
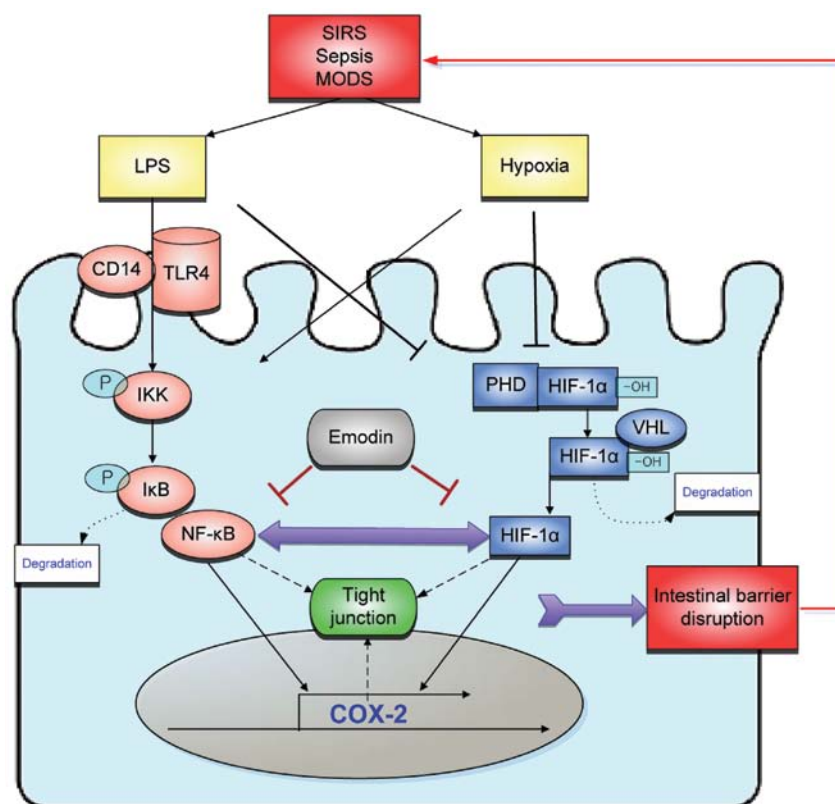
Figure 8. Emodin protects intestinal barrier function by blocking the hypoxia and inflammatory signaling pathways. Lipopolysaccharide (LPS) activates Toll-like receptors and CD14. This response activates IKK, which can then phosphorylate IκB-α, which in turn results in the degradation of IκB-α. Nuclear factor (NF)-κB translocates to the nucleus and regulates the downstream target gene, cyclooxygenase (COX)-2. In the presence of oxygen, prolyl hydroxylase domain protein (PHD) hydroxylates hypoxia-inducible factor (HIF)-1α proline residues and a VHL ubiquitin-protein ligase complex binds, leading to ubiquitination and subsequent proteasomal degradation. Under conditions of hypoxia or inflammation, hydroxylation is inhibited, HIF-1α accumulates, translates to the nucleus and dimerizes with HIF-1β. HIF-1 can regulate transcription of target gene COX-2. All inflammatory factors may destroy the intestinal barrier dysfunction by damaging the tight junction (TJ). Loss of intestinal barrier may worsen the intestinal and systemic disease, lead to SIRS, gut-origin sepsis and multiple organ dysfunction syndrome (MODS). As we known, the critical illness cause decrease in blood flow of gut and increase in intestinal bacteria and their product translocation. A feed-forward loop probably exists between intestinal barrier dysfunction and systemic disease. Emodin can inhibit HIF-1 and NF-κB signaling pathways and protect intestinal barrier functionby preserving TJ.

understood. A recent study demonstrated that HIF-2α plays a unique role in colitis through the regulation of the creatine/creatine kinase shuttle (51). Moreover, it has been reported that HIF-1β is relevant to the pathophysiology of colitis (52). A deeper understanding of how HIFs are uniquely modulated and the mechanisms through which they regulate their downstream target genes in hypoxic and inflammatory stress conditions is essential and requires further investigation.

As a transcription factor, NF-κB plays an essential role in inflammation and innate immunity. It is interesting to note that both the HIF-1α and NF-κB pathways are regulated by inflammatory mediators, as well as by hypoxia (53,6,54). Inflammatory stimuli activate the HIF pathway through transcriptional upregulation of HIF-1 mRNA expression in an NF-κB-dependent manner (55). Conversely, NF-κB activity has been reported under hypoxic conditions to be subject to regulation by HIF-1α (56). It is possible that NF-κB and HIF-1 ultimately determine the magnitude and profile of downstream target genes together in the hypoxic and inflammatory microenvironments.

Hypoxia and inflammation share an interdependent relationship. Both animal and human studies have indicated that hypoxia elicits tissue inflammation, so-called hypoxia-elicited inflammation (57-60). Similarly, during inflammation, such as inflammatory bowel disease, the inflammatory organ becomes ischemic, so-called inflammatory hypoxia or inflammation-associated tissue hypoxia (61-64).

COX-2 and other key proinflammatory genes are transcriptional in a manner that is both HIF-1 and NF-κB dependent (28). A number of studies have revealed an important role for COX-2 in intestinal inflammation. Short *et al* demonstrated that COX-2 is a key enzyme to produce of inflammatory prostanoids in intestinal barrier dysfunction during peritonitis *in vivo*, which was induced by LPS or cecal ligation and puncture (27). The increase in COX-2 expression caused intestinal epithelium injury, increased permeability and bacterial translocation, and internalized TJs. Moreover, certain studies have reported COX-2 inhibitors provide protection from intestinal inflammation (65,66). Consistent with previous studies, our data demonstrated that COX-2 expressoin was induced when the intestinal cells were exposed to LPS, hypoxia $\pm$ LPS, and HR $\pm$ LPS, and it was regulated by both NF-κB and HIF-1α *in vitro*. Different stimuli induced the two signaling pathways at various levels that influence COX-2 to varying degrees. In the cells treated with LPS, the trend in the expression of COX-2 was similar to that of HIF-1α. In the cells exposed to HR, the trend in COX-2 expression was similar to that of NF-κB. In

the cells exposed to hypoxia + LPS and HR + LPS, all the factors showed similar trends in expression. Furthermore, the decreased in TEER and the increase in the FITC-dextran flux following exposure to inflammatory and hypoxic conditions indicated the loss of intestinal barrier function. This suggests the existence of an intriguing link between the HIF-1and NF-κB signaling pathways and shows a unique role for COX-2 in intestinal barrier integrity. Of note, certain studies have indicated that other HIF-responsive genes, such as vascular endothelial growth factor (VEGF), creatine kinase, myosin light chain kinase are also involved in this process (51,67-69). Overall, the hypoxic and inflammatory stress response may activate different signaling pathways in distinct pathological conditions in varying degrees. Further investigation of these pathways may lead to a better understanding of the pathogenesis of hypoxia and inflammation-related diseases.

In this study, we demonstrated that emodin attenuated intestinal epithelial barrier dysfunction caused by LPS and HR *in vitro*. Emodin alleviated the decrease in TEER and the increase in paracellular permeability, and preserved ZO-1 expression (TJ barrier) in Caco-2 intestinal epithelial monolayers. Emodin has been used in the treatment of severe acute pancreatitis/systemic inflammatory response syndrome for thousands of years in China. Emodin has been shown to have anti-inflammatory and antitumor activity *in vivo* and *in vitro* (70,71). The molecular mechanisms through which emodin ameliorates the intestinal epithelial barrier dysfunction induced by LPS and HR are currently unknown. In this study, we demonstrated that emodin inhibited the activation of both the NF-κB and HIF-1α signaling pathways in Caco-2 monolayers exposed to LPS and HR. Thus, we suggest that the inhibition of the NF-κB and HIF-1α signaling pathways may be one of the molecular mechanisms through which emodin attenuates intestinal barrier dysfunction caused by LPS and HR. However, further studies are required to identify other potential mechanisms.

In conclusion, the present study demonstrates that emodin attenuates intestinal barrier dysfunction elicited by LPS and HR by inhibiting the NF-κB and HIF-1α signaling pathways. This may be one of the molecular mechanisms responsible for the protective effects of emodin against intestinal epithelial barrier dysfunction triggered by inflammation and hypoxia. Thus, targeting the restoration of intestinal barrier function is a worthwhile therapeutic strategy for sepsis and other critical illnesses. Hopefully, the results of the present study may lead to the development of novel therapies that may improve clinical outcomes and prognosis of patients with gut-derived sepsis.

Acknowledgements

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