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Original Article

Heme oxygenase-1 enhances cell-cell contact by upregulating tight junctions proteins in Caco-2 cells

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Abstract: Recent studies disclosed that the induction of heme oxygenase-1 (HO-1) expression plays a critical protective role in intestinal damage models. This indicates that activation of HO-1 may be involved in an endogenous defensive mechanism by reducing inflammation and tissue injury in the gastrointestinal tract. In this study, we investigated the role of HO-1 on cell-cell connections, cell motility, and the intracellular distribution of endogenous junctional proteins. We tested the alteration of Claudin-4, Occludin and ZO-1 expression in the hypoxia environment. We measured the gene expression after HO-1 overexpression in this condition. We also did the immunofluorescence experiment to see the expression of these proteins under microscope. After Caco-2 cells was cultured for 2 h, 4 h, 8 h in the hypoxia environment, Claudin-4 and HIF-1α robustly increased in a time dependent manner, but Occludin and ZO-1 significantly decreased. With HO-1 overexpression by virus infection, the expression level of Occludin and ZO-1 were rescued in the hypoxia condition. The Caco-2 cells with control virus infection showed the similar expression panel with the control cells under hypoxia condition. In the immunofluorescence assay, under normal condition, the Occludin and ZO-1 proteins mostly distributed along the cell membrane and closely arranged as a linear with smooth edge. Hypoxia environment disrupted this structure, making these two proteins expressed in a non-close manner. HO-1 overexpression could rescue this type of derangement. Our study showed that HO-1 protein could upregulate the expression of Claudin-4 and ZO-1 proteins and enhance the cell-cell contact.

Keywords: Heme oxygenase-1, Caco-2 cells, cell-cell junction, Occludin, Claudin-4

Introduction

Intestinal ischemia/reperfusion (IR), while clinically not as common as myocardial or cerebral IR, is associated with a 60-80% mortality rate [1-3]. Mesenteric ischemia induces cellular damage, which can be exacerbated by reperfusion, and resulting in tissue injury, systemic inflammation, and even death. Tight junctions (TJs) are the important component of the intestinal mucosa barrier, which encircle each epithelial cell around its apical pole. Not only TJs help maintain cell polarity, but also prevent free interchange of substances. Three integral proteins are found within the TJs: Occludin, the claudin family, zonula occludens (ZO-1), and junction adhesion mole (JAM). Claudin family proteins are major components of TJ strands [4, 5]. The expression of each claudin varies among tissues and developmental status. Studies have shown that Claudin-4 may create charge-selective channels in the paracellular space, and combine with claudin-1/-3 in TJs to enhance cell-cell contact in renal tubular epithelial cells [6, 7]. In addition, Claudin-4, as one of the tight junction protein, not only has the structural function, but also plays an important role in the regulating process [8-11]. Char-lie Wray [12] found that the expression of Claudin-4 gene could protect alveolar epithelial barrier by the tight junction function and also improve alveolar clearance rate to eliminate pulmonary edema. Conversely, inhibition of Claudin-4 gene expression by siRNA would have the opposite effect. Nina A. Hering et al [12] found TGF-β could enhance the intestinal mucosal barrier function by upregulating Claudin-4.

Heme oxygenase-1 (HO-1, HSP-32) is a highly conserved stress-responsive protein, which non-selectively protects normal and cancerous organs, tissues, and cells, including intestinal mucosa barrier, from harmful stimulations and pathological processes [13, 14]. HO-1 functions

as the rate-limiting enzyme in the heme degradation pathway [15, 16], that can be induced by inflammatory cytokines, oxidation, ischemia, hypoxia, and endotoxins [17]. Our previous study has shown that restoration of HO-1 expression promotes Caco-2 cell proliferation *in vitro* [15]. HO-1 also affects expression levels of hypoxia inducible factor-1 gene (HIF-1) *in vitro,* which is a transcription factor regulating hypoxia signal transduction [16].

In this study, we hypothesize that HO-1 protein may involve in the regulation of Claudin-4 expression and play a significant role in protection of intestinal barrier against damages caused by ischemia.

Materials and methods

Cell culture

The Caco-2 cell line was obtained from American Type Culture Collection (Manassas, VA, USA) and was maintained in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Invitrogen Ltd., Carlsbad, CA, USA) containing 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 10% (v/v) fetal bovine serum (FBS; GIBCO) that had been heat-inactivated for 30 minutes at 56°C, 100 U/ml penicillin, and 50 μg/ml streptomycin, and incubated at 37°C under 5% CO_2 air atmosphere. Cells between passages 17 and 25 were used.

PCR amplification of the HO-1 gene

A pair of PCR primers was designed using Primer 5.0 software according to the published sequence of human genome in GenBank (NM_002133), including 5'-TTAAGATCTATGGAGCGTCCGCAACC-3'; 5'-CTGAAGCTTTCACATGGCATAAAGCCCTAC-3'. The primers contained BglII and HindIII restriction sites at their 5'-termini, respectively. Total human genomic DNA was extracted from intestinal tissue. Reactions were run in a thermocycler (Techgene, UK).

Recombinant adenovirus plasmid construction

The product of PCR amplification of the HO-1 gene was inserted into pMD18-simple T vector (TaKaRa, Japan) and then cloned into the transfer vector pShuttle-CMV (Qbiogene, USA) using BglII and HindIII (BioLabs, UK); the resulting vector was designated pShuttle-CMV-HO. The recombinant plasmid was identified by PCR, BglII, and HindIII restriction enzyme digestion and sequencing. The plasmid was then linearized with PmeI (BioLabs, UK) using the pAdEasy-1 skeleton vector (Stratagene, CA, USA) with a Bio-Rad Gene Pulser at 200, 2.5 kV, and 25 F conditions for recombination; the plasmid was designated pAd-HO. The recombinant plasmid was obtained by selecting blue colonies and identified by PCR and PacI digestion (BioLabs, UK).

Transfection and isolation of the recombinant adenovirus

To obtain recombinant adenovirus, human embryonic kidney 293 (HEK293) cells were cultured in DMEM (Invitrogen) supplemented with 3.7 g sodium bicarbonate/l and 10% FBS (Hyclone, USA). The recombinant adenovirus plasmids were linearized with PacI and then mixed with Lipofectamine 2000 transfection reagent (Invitrogen). The cells were incubated with 5% CO_2 at 37°C for 3-7 days. The virus, designated rAd-HO, was propagated in HEK293 cells, collected at the appearance of a cytopathic effect. The titer of the virus was determined by the method of 50% tissue culture infectious dose (TCID50) after passage in HEK293 cells.

Cell transfection

For transduction, Caco-2 cells were infected with either control lentivirus or lentivirus overexpression HO-1. After 48 hours of transduction, the cells were harvested and prepared for subsequent studies. Cells were plated in 24-well plates (2×10^4 cells/well) overnight. The lentiviruses were diluted in 0.2 ml (10^8 TU/ml) complete medium containing polybrene (5 μg/ml) and added to the cells for 12 hours incubation at 37°C, followed by incubation in 0.3 ml of freshly prepared polybrene-RPMI 1640 for another 24 hours, which was then replaced with fresh RPMI 1640 medium and the cells were cultured for a further 48 hours period. The level of HO-1 expression in the cells was assayed by western blotting and qPCR 48 hours after transfection.

Western blotting

Cells were lysed in M-PER Mammalian Protein Extraction Reagent (Pierce, Rockford, IL, USA)

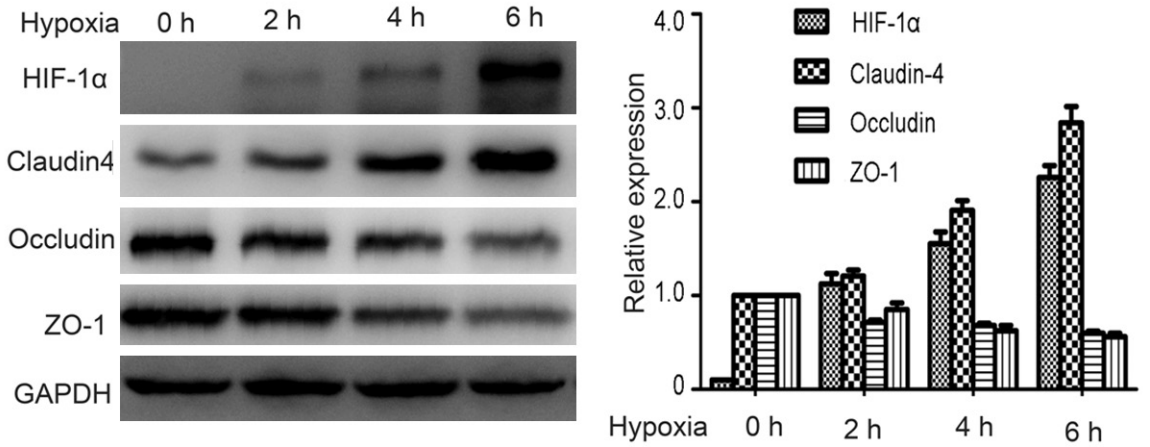

Figure 1. Claudin-4 and HIF-1α protein were significantly upregulated with time dependent (P<0.01) and Occludin decreased significantly compared to normal group (P<0.05).

containing a cocktail of proteinase inhibitors (Bio-Rad). The lysed proteins were quantified using a bicinchoninic acid protein assay kit from Pierce. Subsequently, equal amounts of proteins were separated by SDS-PAGE, and then transferred to polyvinylidene difluoride membranes (Bio-Rad). The blots were washed with TBST and then incubated with a specific primary antibody overnight at 4°C. The blots were again washed with TBST and then incubated with horseradish peroxidase-conjugated anti-rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 hours at room temperature. Proteins were visualized with an enhanced chemiluminescence detection system (Amersham, Freiburg, Germany). Autoradiograms were quantified by densitometry (Quantity One software, Bio-Rad). As a loading control, GAPDH-specific antibody (Sigma, St. Louis, MO, USA) was used. Polyclonal antibodies for Claudin-4, HIF-1α, Occludin and ZO-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and monoclonal antibody for HO-1 (Abcam, Shanghai, China) were utilized in this study.

Immunofluorescence

Caco-2 cells were seeded in a 35-mm dish containing a coverslip coated with poly-D-lysine. For permeabilized staining, 24 hours post-seeding cells were fixed in 4% paraformaldehyde for 15 minutes and permeabilized with methanol at 4°C. The cells were incubated in 5% skimmed milk/PBS containing a 1:100 dilution of mouse anti-HO-1 antibody at room temperature for 45 minutes. The cells were then washed in PBS three times followed by incubation with Cy3-conjugated donkey anti-mouse IgG at room temperature for 30 minutes. Then, the cells were washed in PBS three times followed by incubation with DAPI. The slides were mounted and observed under a fluorescent microscope.

Statistical analysis

Gene expression levels (mean ± standard deviation) from three separate experiments were calculated using SPSS 16.0 statistical analysis software (SPSS Inc., USA). Differences between groups were analyzed. A value of $P<0.05$ was considered statistically significant.

Results

Protein expression in Caco-2 cells under hypoxia condition

Caco-2 intestinal cells can transform into normal intestinal epithelium cells, and Caco-2 cell monolayers have been well established as an *in vitro* model for the study of the normal intestinal epithelium as an intestinal barrier. Caco-2 cells were cultured in a hypoxia condition (1% O_2) for 2 h, 4 h and 8 h in the study, the relative expression levels of HIF-1α, claudin-4, Occludin and ZO-1 proteins were shown in **Figure 1**. Our results found that the expression of HIF-1α and claudin-4 proteins were significantly increased in a time-dependent manner, compared to control group (**Figure 1**, P<0.01). However, the expression of Occludin and ZO-1 decreased sig-

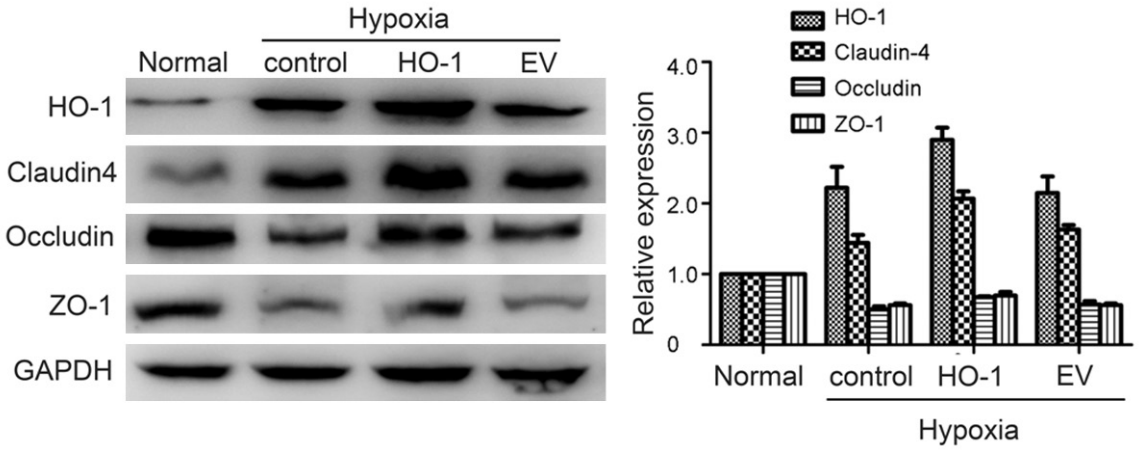

Figure 2. In the hypoxia group, the levels of Occludin and ZO-1 were less than normal group (P<0.01), but claudin-4 expression was higher than normal oxygen group (P<0.01). In the HO-1 gene transfection group, HO-1, claudin-4, Occludin and ZO-1 were higher than them in hypoxia group (P<0.05). However there were no difference in the levels of them between in hypoxia group and in empty plasmid transfection group (P>0.05).

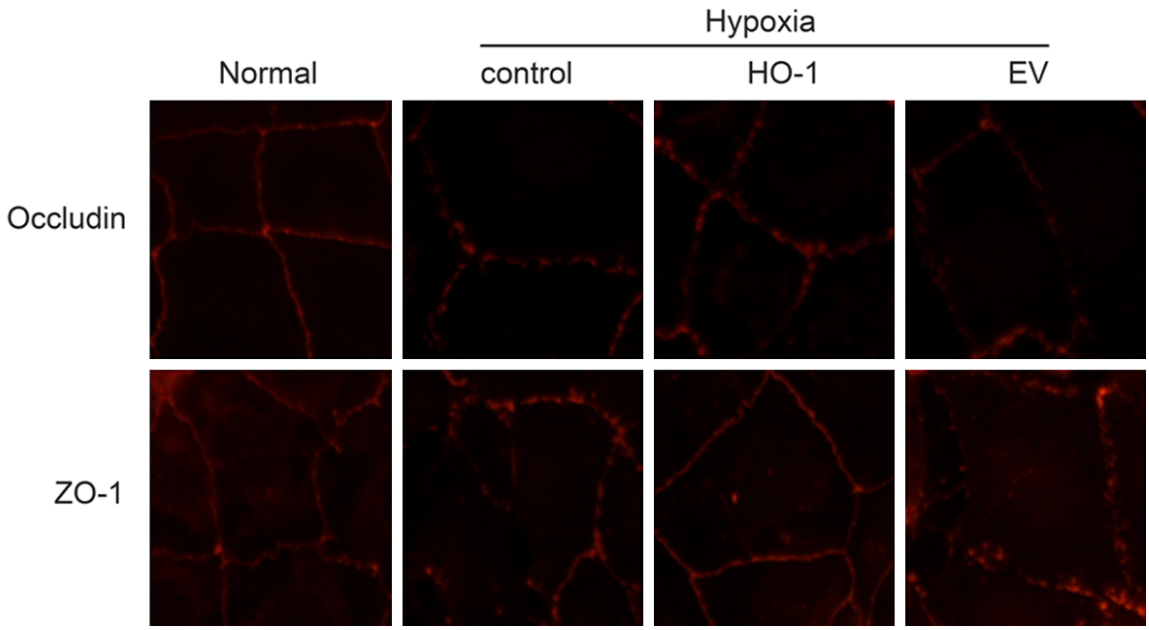

Figure 3. Occludin and ZO-1 often distributed along the cell membrane and closely arranged as a linear with smooth edge in the normal groups; their edge were rough or even collapsed with gaps between cells in the hypoxia group and empty plasmid transfection group; the damage was slight and the edge was continuous in the HO-1 gene transfection group.

nificantly, compared to control group (**Figure 1**, P<0.05).

The changes of protein expression after transfection HO-1 gene over-expression vector

We have shown that hypoxia affected the relative expression of HIF-1α, claudin-4, Occludin and ZO-1 protein in Caco-2 cells (see above). Next, Caco-2 cells were cultured in 1% O_2 by restoring HO-1 expression for 8 h. In the hypoxia group the expression of Occludin was less than control group (**Figure 2**, P<0.01), but claudin-4 expression was higher than control group (**Figure 2**, P<0.01). In the HO-1 gene transfection group, HO-1, Claudin-4, Occludin and ZO-1

were higher than them in hypoxia group (**Figure 2**, P<0.05).

The position of Occludin and ZO-1 genes with immunofluorescence

To test whether HO-1 overexpression affected the position of Occludin and ZO-1 with immunofluorescence in different condition in Caco-2 cells. Successful transfection was determined by a red fluorescence, our results showed that Occludin and ZO-1 often distributed along the cell membrane and closely arranged as a linear with smooth edge in the normal groups; their edge were rough or even collapsed with gaps between cells in the hypoxia group and empty plasmid transfection group; the damage was slight and the edge was continuous in the HO-1 gene transfection group under immunofluorescence (**Figure 3**).

Discussion

The intestinal mucosal barrier is an important defensive barrier of human. Intestinal mucosal barrier comprises mechanical barrier, biological barrier, chemical barrier and immune barrier, of which mechanical barrier is the most important one [18, 19]. Tight junctions (TJs) are essential for normal intestinal mucosal barrier function. TJ proteins, such as ZO-1, claudin, Claudin-4 and Occludin, are indispensable to maintain the function of TJs [20-23]. In pathological conditions, hypoxia is a common mechanism leading to the dysfunction of intestinal mucous barrier. Some studies show that intestinal mucosal barrier injury and intestinal permeability enhancement are early pathophysiological change of IRI [24, 25]. Intestinal epithelial cell layer is an important physiological barrier to maintain a stable environment within the bowel, preventing intestinal invasion of harmful substances. In recent years, more and more research focused on the integrity of tight junction proteins, and intestinal mucosal barrier permeability may be the pathological basis of serious intestinal disorders including inflammatory bowel disease [24, 26]. Therefore, this research aimed to further explore how to hence cell-cell contacts to protect intestinal mucosal barrier.

Although the sequence of development of intestinal mucosal injury after Ischemia/reperfusion has been studied intensively; most studies have focused on the relatively late phase. On the other hand, grafting of the small intestine has attracted a lot of attention in recent years. The transplantation is an effective strategy for treating patients with severe intestinal failure. However, not only organ rejection but also early ischemia/reperfusion injury must be overcome in order to succeed with the transplantation. We used an *in vitro* system for assessing early ischemia/reperfusion injury using monolayers of human intestinal epithelial cell line Caco-2, in which lipid peroxidation chemically caused by *tertiary*-butylhydroperoxide (t-BuOOH) acts as a trigger of the injury. Using this model, we showed that in the hypoxia group, the level of Occludin and ZO-1 expression in Caco-2 cells was less than the control group, but the expression of claudin-4 and HIF-1α was higher than the control group. Meanwhile, HO-1, Claudin-4, Occludin and ZO-1 in the HO-1 gene transfection group were higher than that in hypoxia group. But there were no difference in the levels of them in empty plasmid transfection group. The results suggest that HO-1 can reduce the injury of hypoxia on the intestinal mucosal barrier. To further validate our guess that HO-1 protein can enhance cell-cell contact by upregulating Claudin-4 in Caco-2 cells. The protective mechanism of HO-1 on intestinal mucosal barrier may be related to heme degradation product (ferrousion, biliverdin and CO) [27-29]. Chelate ferrous ion combined with protein is a powerful antioxidant; Biliverdin was transformed into bilirubin by enzyme catalysis which could remove oxygen free radicals and reduce cell injury by lipid over-oxidation. Philippe Pinton [30] found that activation of MAPKs can promote the expression of Claudin-4 gene, resulting in the high expression of Claudin-4.

To sum up, on the basis of the successful establishment of cell model, we constructed over-expression vector of HO-1 gene and successfully obtained the cell lines of HO-1 gene over-expression. Confirm that HO-1 protein can enhance cell-cell contact by upregulating Claudin-4 in Caco-2 cells. But the specific interaction mechanism is not very clear. Therefore, our study will focus on the molecular mechanism among them next step. We also will get the results of the experiments in vitro back to animal experimental model for further verification.

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Disclosure of conflict of interest

None.

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