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Interferon-γ-Induced Intestinal Epithelial Barrier Dysfunction by NF-κB/HIF-1α Pathway

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Interferon-γ (IFN-γ) plays an important role in intestinal barrier dysfunction. However, the mechanisms are not fully understood. As hypoxia-inducible factor-1 (HIF-1) is a critical determinant response to hypoxia and inflammation, which has been shown to be deleterious to intestinal barrier function, we hypothesized that IFN-γ induces loss of barrier function through the regulation of HIF-1α activation and function. In this study, we detected the expressions of HIF-1α and tight junction proteins in IFN-γ-treated T84 intestinal epithelial cell line. IFN-γ led to an increase of HIF-1α expression in time- and dose-dependent manners but did not change the expression of HIF-1β. The IFN-γ-induced increase in HIF-1α was associated with an activation of NF-κB. Treatment with the NF-κB inhibitor, pyrolidinedithiocarbamate (PDTC), significantly suppressed the activation of NF-κB and the expression of HIF-1α. In addition, IFN-γ also increased intestinal epithelial permeability and depletion of tight junction proteins; inhibition of NF-κB or HIF-1α prevented the increase in intestinal permeability and alteration in tight junction protein expressions. Interestingly, we demonstrated that a significant portion of IFN-γ activation NF-kB and modulation tight junction expression is mediated through HIF-1α. Taken together, this study suggested that IFN-γ induced the loss of epithelial barrier function and disruption of tight junction proteins, by upregulation of HIF-1α expression through NF-κB pathway.

Introduction

The main function of the human intestinal epithelia is to allow the absorption of nutrients while also functioning as a barrier, which prevents foreign antigens and pathogens entering the mucosal tissues and potentially causing disease. Tight junctions and its associated proteins, including zonulaoccludens, occludin and claudins, and so on, are theapicalmost adhesive junctional complexes and act as a structural and functional barrier against paracellular permeation of luminal substances (Groschwitz and Hogan 2009; Ulluwishewa and others 2011). A breakdown or impairment of the epithelial barrier has been implicated as a critical determinant in the predisposition to intestinal inflammation and a number of inflammatory disorders, such as inflammatory bowel disease (Suenaert and others 2002), food allergy (Ventura and others 2006), celiac disease (Diraimondo and others 2012). Although the mechanisms of intestinal barrier dysfunction involved in these diseases are incompletely understood, proinflammatory cytokines have been shown to play important roles in these processes. For example, interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α) and lipopolysaccharide (LPS) as well as interleukins (IL), which are central mediators of intestinal inflammatory diseases, induce the loss of intestinal epithelial barrier function (Youakim and Ahdieh 1999; Suenaert and others 2002; Bruewer and others 2003; Prasad and others 2005; Wang and others 2005; Ye and others 2006; Al-Sadi and Ma 2007; Wisner and others 2008; Capaldo and Nusrat 2009; Groschwitz and Hogan 2009; He and others 2012; Guo and others 2013). In addition, our previous studies demonstrated that IFN-γ evokes epithelial cell apoptosis and impairs intestinal barrier function (Yang and others 2002, 2003). Although the precise mechanisms involved in intestinal barrier dysfunction induced by proinflammatory cytokines are still unclear, it has been well demonstrated that the NF-κB pathway plays an important role in this cytokine-induced intestinal barrier dysfunction (Boivin and others 2009; Dehne and Brune 2009).

Hypoxia-inducible factor-1 alpha (HIF-1α) has emerged as a central determinant in the pathophysiological response to cellular hypoxia (Semenza 2001, 2007). HIF is a heterodimer of bHLH-PAS proteins and consists of oxygen-regulated alpha subunit (such as HIF-1α) and a constitutively expressed beta subunit (such as HIF-1β, also called ARNT1) that binds DNA at specific locations termed hypoxia response elements (HREs) (Kaelin and Ratcliffe 2008); its activation is

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dependent on the stabilization of an oxygen-sensitive degradation pathway that is regulated by a family of prolyl hydroxylase (PHD) enzymes. Previous studies have indicated that HIF-1α plays an important role in the majority of clinically relevant hypoxic/ischemic episodes and human cancers (Toiyama and others 2010; Nam and others 2011; Hiraki and others 2012). Recent studies have showed that HIF-1α activation is deleterious to intestinal barrier function associated with hypoxia, ischemia/reperfusion, and inflammation (Rosenberger and others 2007; Feinman and others 2010; Kannan and others 2011; Liu and others 2011).

Recently, induction of *HIF-1α* by proinflammatory cytokines, not only under hypoxia but also under normoxic conditions, has been described in numbers of studies (Jung and others 2003; Frede and others 2005; Belaiba and others 2007; Peyssonnaux and others 2007). Many of the stimuli that induce *HIF-1* in normoxia are known to activate a number of other transcription factors such as NF-κB (Taylor 2008). In addition, the *HIF-1α* promoter contains an active NF-κB binding site in a position −197/188 upstream of the transcription start site (van Uden and others 2008). Importantly, studies have demonstrated that the NF-κB can be activated by IFN-γ (Gough and others 2008; Lin and others 2012).

Since IFN-γ and HIF-1α play important roles in intestinal barrier dysfunction, and NF-κB could be activated by IFN-γ, we asked the relationship of these 2 factors in this process. For this purpose, we aimed to clarify if epithelial-derived HIF-1α activation and function are regulated by IFN-γ. Our data indicated that IFN-γ activates the NF-κB pathway, which, in turn, leads to the activation of HIF-1α; and HIF-1α play a major mechanistic role in IFN-γ-induced intestinal barrier dysfunction. Taken together, our finding, for the first time, reveals that IFN-γ induces intestinal epithelial barrier dysfunction through NF-κB/HIF-1α pathway.

Materials and Methods

Materials

IFN-γ was purchased from Novoprotein. HIF-1α inhibitor YC-1 was purchased from Sigma. The inhibitor of NF-κB pyrolidinedithiocarbamate (PDTC), Nuclear and Cytoplasmic Protein Extraction Kit, and anti-H3 antibody were purchased from Beyotime. Anti-p65, anti-IκBα, anti-P-IκBα, anti-ZO-1, and anti-Occludin antibodies were purchased from Santa Cruz Biotechnology, Inc. Anti-GAPDH antibody was purchased from Goodhere Biotechnology. Anti-HIF-1α, anti-HIF-1β, anti-Claudin-1, anti-Claudin-2 antibodies were purchased from Abcam or Proteintech.

Cell culture

T84 intestinal epithelial cells were purchased from China Center for Type Culture Collection and maintained at 37°C in 1:1 DMEM/Ham's F-12 medium supplemented with10% fetal bovine serum (Gemini Bioproducts) and 100 U/100 μg/mL penicillin and streptomycin (Invitrogen). The cells were kept at 37°C in a 5% CO_2 environment. Culture medium was changed every 2 days. T84 cells were subcultured after partial digestion with 0.25% trypsin and 0.53 mM EDTA in Hank's balanced saline solution (HBSS) without Ca^{2+} and Mg^{2+}. Unless stated otherwise, in all experiments, IFN-γ was added to the basolateral compartment only and control monolayers were incubated with the cell culture medium.

RNA isolation and real-time quantitative RT-PCR

Total RNA was isolated using a Trizol reagent (TaKaRa) according to the manufacturer's protocol and quantified by NanoDrop 2000 (Thermo). Reverse transcription of the RNA was performed by using a PrimeScript® RT reagent kit (TaKaRa) according to the manufacturer's instructions. Real-time quantitative RT-PCR (qRT-PCR) was performed on 50 ng cDNA template, using Rotor-Gene Q (Qiagen) and the SYBR® Premix Ex TaqTM II (TaKaRa), in a total volume of 20 μL. The PCR was performed as follows: denaturation at 94°C for 10 s, annealing at 60°C for 15 s, and elongation at 72°C for 20 s, for a total of 40 cycles. Primer sequences used in this study are designed as follows: *HIF-1α* (forward 5′-GCCGCTGGAGA CACAATCATA-3′ and reverse 5′-GGTGAGGGGAGCATTA CATCAT-3′) and *β-actin* (forward 5′-CCACGAAACTACCTT CAACTCC-3′ and reverse 5′-GTGATCTCCTTCTGCATC CTG-3′). All data were normalized using *β-actin* as reference values and expressed as relative fold increases over control. The amplification efficiency of *HIF-1α* and *β-actin* was ∼1.72 and the difference between them was lower than 0.02.

Measurement of transepithelial electrical resistance

Transepithelial electrical resistance (TER) values of monolayers grown on 0.33 cm^2 Transwell supports were determined with a Millicell-ERS voltohmmeter (Millipore), and TER measurements were calculated in Ohms cm^2 ($\Omega(cm^2)$) to determine the TER of the epithelium alone (Wells and others 1998). To facilitate comparisons between conditions, TER was normalized to initial value, and expressed as percentage of initial resistance values.

Western blot analysis

The cells were washed twice with phosphate-buffered saline (PBS) before lysis in cold RIPA buffer (PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 μg/mL APMSF, 1.0 mM sodium orthovandate, 1×mammalian protease inhibitor cocktail; Sigma-Aldrich). Where indicated, nuclear epithelial cell fractions were prepared using the Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime) according to the manufacturer's instructions. The protein concentration was determined according to the Bradford method using BCA assay reagent (Beyotime). Samples (25 μg protein) were loaded onto 8–12% SDS-PAGE, and the proteins were then electrophoretical transferred to a polyvinylidene fluoride membrane blocked by 5% bovine serum albumin (BSA) for 1 h at room temperature and then incubated with antibodies overnight at 4°C: anti-p65 (1:200), anti-IκBα (1:200), P-IκBα (1:200), H3 (1:1,000), ZO-1 (1:500), Occludin (1:500), HIF-1α (1:1,000), HIF-1β (1:2,000), Claudin-1 (1:1,000), Claudin-2 (1:1,000), and anti-GAPDH (1:1,000). The membranes were then washed 3 times in TBST (50 mM Tris-HCl pH7.5, 140 mM NaCl, 0.1% Tween) and incubated with secondary antibody at room temperature for 1 h. A enhanced chemiluminescence reagent, ECL Western blotting detection reagent (Boster), was used to make the labeled protein bands to be detected with Image Station 4000R (Kodak).

Fluorescence microscopy

HIF-1α and cellular tight junction proteins: ZO-1, Occludin, and Claudin-1 were assayed with an immunofluorescent

technique. T84 monolayers were washed twice with PBS before fixed with 4% paraformaldehyde for 15 min at room temperature, washed again, and permeabilized in for 10 min with 0.1% Triton X-100. The cells were blocked by 5% solution of BSA for 30 min at room temperature and then incubated with anti-HIF-1α (1:25), anti-ZO-1 (1:100), Occludin (1:100), and Claudin-1 (1:200) antibodies diluted in PBS containing 5% BSA at 4°C overnight; monolayers were washed with PBS and incubated with Cy3 red-conjugated goat anti-rabbit secondary antibodies (1:500; Boster) for 1 h in dark conditions. They were then washed and mounted using ProLong® Gold antifade reagent with DAPI (Invitrogen). Negative controls were also carried out omitting the primary antibody. Monolayers were imaged using a laser scanning fluorescence microscopy (Leica TCS SP5; Leica).

Statistical analyses

Statistical analyses were performed using SPSS 13.0 software. All experimental data are shown as mean ± SD. Comparisons among 3 or more groups were made by analysis of variance (ANOVA), and the 2 groups were compared by Student's *t*-test. A P value less than 0.05 was considered statistically significant in all cases. All reported significance levels represent 2-tailed P values. If not otherwise stated, all experiments were repeated at least 3 individual experiments to ensure reproducibility.

Results

IFN-γ induces HIF-1α expression in time- and dose-dependent manners

Hypoxia-inducible factor 1 (HIF-1) is one of the master regulators that orchestrate the cellular responses to hypoxia. Even if the HIF-1 system evolved exclusively for the adaptation of organisms to hypoxia, recent studies show that both HIF-1α protein abundance and HIF-1α transactivation activity can also be induced under normoxic conditions in response to various nonhypoxic stimuli, such as TNF-α and IL-1β as well as LPS (Jung and others 2003; Frede and others 2005; Peyssonnaux and others 2007). Moreover, these stimulating factors play vital roles in the modulation of intestinal barrier function disruption. Similarly, it has been shown that IFN-γ also plays a deleterious role in intestinal barrier function (Youakim and Ahdieh 1999; Utech and others 2005; Juuti-Uusitalo and others 2011). We hypothesized that one mechanism by which IFN-γ mediates a loss of intestinal barrier function is by the activation of HIF-1α. To address this, the effect of IFN-γ on HIF-1α accumulation in intestinal epithelial cells was investigated. T84 epithelial cells were treated with 50 ng/mL IFN-γ or vehicle and then subjected to normoxia or hypoxia for 6 h. HIF-1α protein levels were examined by Western blot. As shown in Fig. 1A, there is no significant change of HIF-1α expression of T84 epithelial cells after 6 h treatment with IFN-γ, whereas hypoxia treatment for 6 h led to a dramatic increase of HIF-1α protein expression compared with controls. The HIF-1α protein expression of T84 cells receiving hypoxia stimulation increased more significantly than that receiving IFN-γ stimulation (Fig. 1A). The mechanism for this difference is not clear. It maybe that IFN-γ-induced increase in HIF-1α is a relatively slow process, potentially requiring a more prolonged treatment of 24 h or more (Gerber and others 2009).

Based on the results presented above, we next examined whether IFN-γ-induced HIF-1α are synchronized in time. T84 were treated with IFN-γ (50 ng/mL) for up to 48 h, and the total RNA and protein were harvested at the indicated times (0, 6, 12, 24, and 48 h). As shown in Fig. 1B, a time-dependent increase of *HIF-1α* mRNA was observed in the presence of IFN-γ. The increase in *HIF-1α* mRNA was not significant at earlier time points (eg, at 6 h) but became significant by 24 and 48 h. We also detected an increase in the levels of HIF-1α protein by Western blot at the indicated times of IFN-γ treatment. HIF-1α protein expression was also induced in a time-dependent (Fig. 1C) manner by IFN-γ with little to no induction occurring until 6 h following cytokine stimulation, after which levels of protein remained increasing for up to 48 h, and the change of HIF-1α protein is consistent with the result of real-time quantitative PCR.

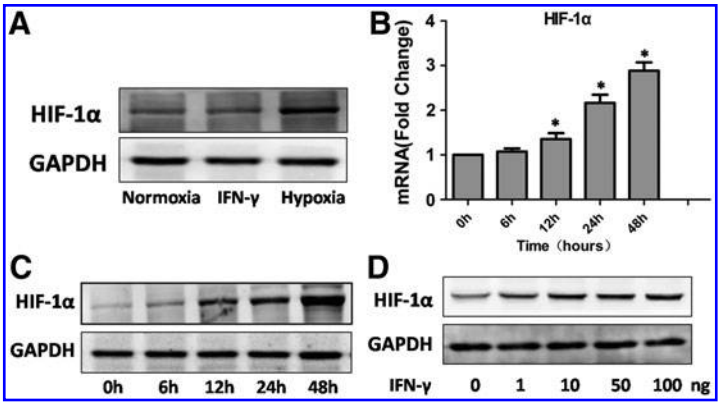

FIG. 1. Changes of hypoxia-inducible factor-1 alpha (HIF-1α) expression in T84 intestinal epithelial cells after interferon-γ (IFN-γ) treatment. **(A)** T84 intestinal epithelial cells were treated with vehicle control, 50 ng/mL IFN-γ, or hypoxia (1% O_2) for 6 h, and HIF-1α protein expression were assayed. No significant changes of HIF-1α protein expression were found after T84 cells treated with IFN-γ for 6 h, whereas hypoxia caused a dramatic increase in HIF-1α protein expression. **(B)** T84 cells were treated with 50 ng/mL of IFN-γ, and IFN-γ caused a time-dependent increase in *HIF-1α* mRNA expression **(B)** and protein expression **(C)**. *HIF-1α* mRNA were calculated relative to *β-actin* and expressed as fold change relative to controls. **(D)** T84 cells were treated with different concentrations of IFN-γ for 24 h, and IFN-γ-induced HIF-1α expressions in a dose-dependent manner. *$P < 0.05$ versus control. Data are representative of 3 similar experiments.

We also detected different concentrations of IFN-γ on the expressions of HIF-1α. In T84 cells, IFN-γ was found to increase HIF-1a in a dose-dependent manner after 24 h of treatment (Fig. 1D).

The expression of HIF-1β in T84 cells is independent of IFN-γ

It has been demonstrated that HIF-1 is a heterodimer of bHLH-PAS proteins and consists of oxygen-regulated alpha subunit and a constitutively expressed beta subunit (Kaelin and Ratcliffe 2008). Because HIF-1α/HIF-1β heterodimerization is required for the stable of HIF-1 and formation of the active transcriptional complex, we examined whether the expression of HIF-1β was changed in IFN-γ-treated T84 cells. As shown in Fig. 2A and B, IFN-γ did not induce any changes of HIF-1β expression in a time- or dose-dependent manner.

As HIF-1, plays biological activities, needs to bind with DNA at specific locations termed HREs, we used immunofluorescence staining to examine whether nuclear HIF-1a was increased after treatment of IFN-γ. As illustrated in Fig. 2C, compared with control, IFN-γ increased the expression of HIF-1a in nuclear, which indicated the activation of HIF-1.

IFN-γ-induced HIF-1α expression is mediated through NF-κB pathway activation

The interactions between proinflammatory cytokines and elements of the HIF system have recently been observed in several studies (Jung and others 2003; Frede and others 2005; Peyssonnaux and others 2007; Hot and others 2012). Cytokines that activate NF-κB may increase HIF-1α expression by driving increased transcription (Rius and others 2008). Recently, several studies have demonstrated that the NF-κB can be activated by IFN-γ (Gough and others 2008; Lin and others 2012). Therefore, to investigate the potential role of the NF-κB pathway in the effects of IFN-γ on T84 intestinal epithelial cells, cells were pretreated with the NF-κB inhibitor PDTC, followed by the addition of IFN-γ or vehicle control. Levels of IκB family proteins and the NF-κB subunit protein p65 were assayed in cytoplasmic and nuclear extracts of T84 monolayers treated with IFN-γ. Figure 3A shows that IFN-γ treatment induced a significant increase in cytoplasmic levels of phosphorylated IκB, with a concomitant decrease in IκB levels. Consistent with this, at 12 h of IFN-γ treatment, nuclear levels of active p65 NF-κB subunit were dramatically increased, both of which indicate robust NF-kB activation, whereas pretreatment with PDTC inhibited IFN-γ-induced nuclear translocation of p65 and decrease of IκB in cytoplasm levels after 12 h of cytokine treatment.

To determine whether IFN-γ-mediated NF-kB signaling is required for the expression of HIF-1α, the NF-κB inhibitor PDTC was added to T84 medium 1 h before IFN-γ treatment. As shown in Fig. 3B, PDTC significantly inhibited IFN-γ-induced HIF-1α protein expression.

Blockade of either NF-κB activation or HIF-1α attenuates IFN-γ-induced intestinal barrier dysfunction

To determine the effect of IFN-γ on T84 intestinal epithelial permeability, TER, a sensitive measure of barrier integrity, was employed. Treatment of the cells with different doses of IFN-γ (0, 1, 10, 50, and 100 ng/mL) added to the basolateral side of the monolayer resulted in a dose-dependent decrease in TER (Fig. 4A). The time course of IFN-γ (50 ng/mL) effect on T84 TER is shown in Fig. 4B. IFN-γ caused a time-dependent drop in T84 TER with a maximal drop occurring at 48 h after treated with IFN-γ (Fig. 4B).

It has been shown that HIF-1α plays an important role in intestinal barrier dysfunction (Rosenberger and others 2007; Feinman and others 2010; Kannan and others 2011; Liu and others 2011). To examine the interdependency of HIF-1α- on IFN-γ-induced intestinal barrier dysfunction, T84 cells was

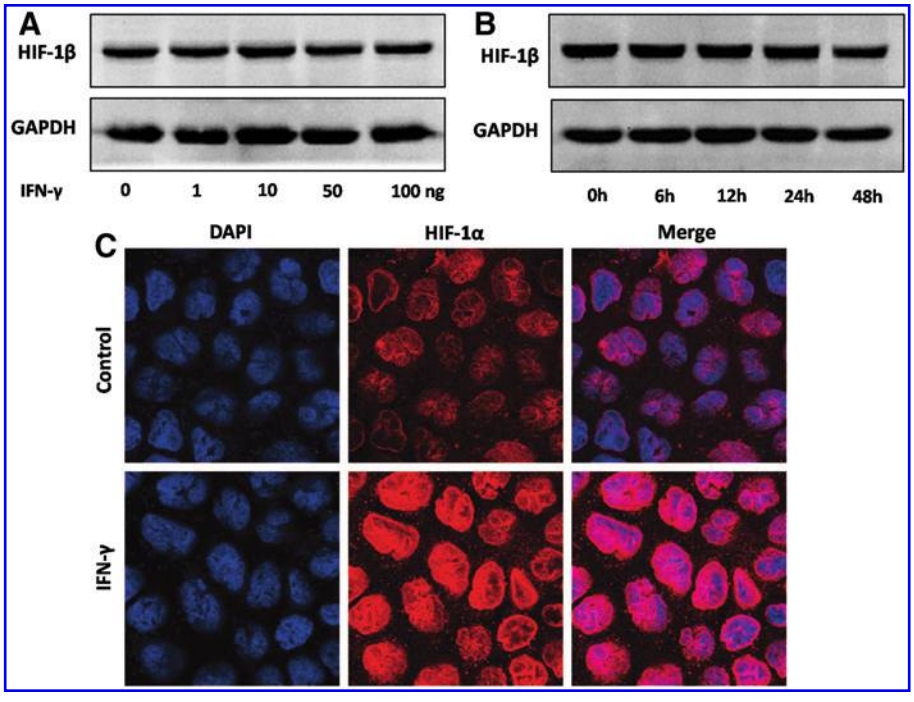

FIG. 2. The expression of HIF-1β in T84 cells is independent of IFN-γ. **(A)** T84 cells were treated with different concentrations of IFN-γ, and HIF-1β protein expressions were assayed. IFN-γ did not significantly change the expression of HIF-1β. **(B)** T84 cells were treated with IFN-γ (50 ng/mL) for 0, 6, 12, 24, and 48 h, HIF-1β is stably expressed with time. **(C)** T84 cells were treated with IFN-γ (50 ng/mL) for 24 h, and HIF-1α was analyzed by immunofluorescence staining. Compared with control, IFN-γ induced significantly increase of HIF-1α in nuclear. Magnification: ×2000.

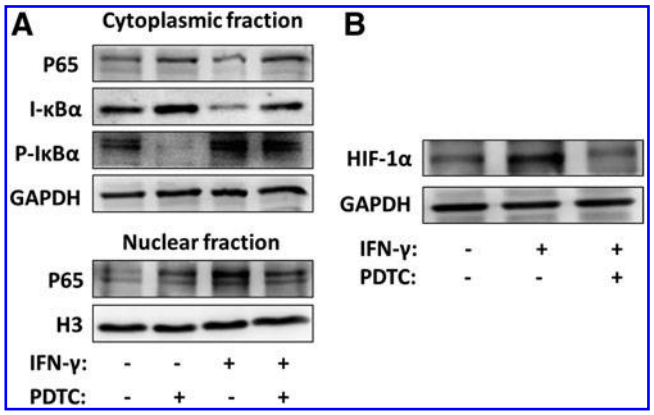

FIG. 3. NF-κB pathway activation is required for IFN-γ to regulate HIF-1α expression. **(A)** T84 cells were treated with IFN-γ (50 ng/mL) for the indicated time, and the ability of IFN-γ to activate the NF-kB signaling pathway was assayed by measuring levels of cytoplasmic proteins (IkB, P-IkB, p65) and nuclear NF-kB (p65) by Western immunoblot. IFN-γ treatment induced translocation of p65 from the cytoplasm to the nucleus in T84 cells. **(B)** T84 cells were treated with 50 ng/mL IFN-γ for 48 h. Treatment with IFN-γ caused a marked increase in HIF-1α protein expression, whereas the NF-κB inhibitor pyrolidinedithiocarbamate (PDTC) prevented the IFN-γ induced increase of HIF-1α expression. Data are representative of 3 similar experiments.

pretreated with YC-1, a novel HIF-1α inhibitor, before IFN-γ treatment. As shown in Fig. 4C, the expression of HIF-1α protein was concentration-dependently inhibited by YC-1 after 48 h treatment. YC-1 can also inhibit the IFN-γ-induced drop in T84 TER (Fig. 4D), indicating that HIF-1α activation was required for the IFN-γ-induced loss of epithelial barrier function.

Blockade of either NF-κB activation or HIF-1α attenuates disruption of tight junction proteins induced by IFN-γ treatment

Previous studies have demonstrated that IFN-γ synergistically disrupts intestinal mucosal morphology and impairs intestinal epithelial barrier function by altering the expression and distribution of tight junction proteins (Youakim and Ahdieh 1999; Bruewer and others 2005; Willemsen and others 2005). In the following studies, we examined the mechanism by which HIF-1α is involved in IFN-γ-induced disruption in tight junction protein. Similar to previous reports, IFN-γ caused significant decrease in the expression of tight junction proteins ZO-1 and Claudin-1 (Fig. 5A). IFN-γ also increased the expression of tight junction protein Claudin-2, which has been shown to induce cation-selective channels in the tight junction of epithelial cells, resulting in increased paracellular permeability (Furuse and others 2001; Amasheh and others 2002). However, no significant changes were measured in Occludin expression after treated with IFN-γ. Importantly, pretreatment with the HIF-1α inhibitor YC-1 significantly attenuates IFN-γ-induced changes of these tight junction protein expressions (Fig. 5B). Similarly, use of the NF-κB inhibitor PDTC also attenuates the change of IFN-γ-induced tight junction proteins (Fig. 5A).

As cytokine-mediated disruption of epithelial tight junctions can lead to either an internalization (ie, Occludin) (Utech and others 2005) or absolute loss of these proteins (ie, ZO-1) (Youakim and Ahdieh 1999), we next used immunofluorescence staining to visualize the tight junction. No staining was visible in any of the negative controls (date was

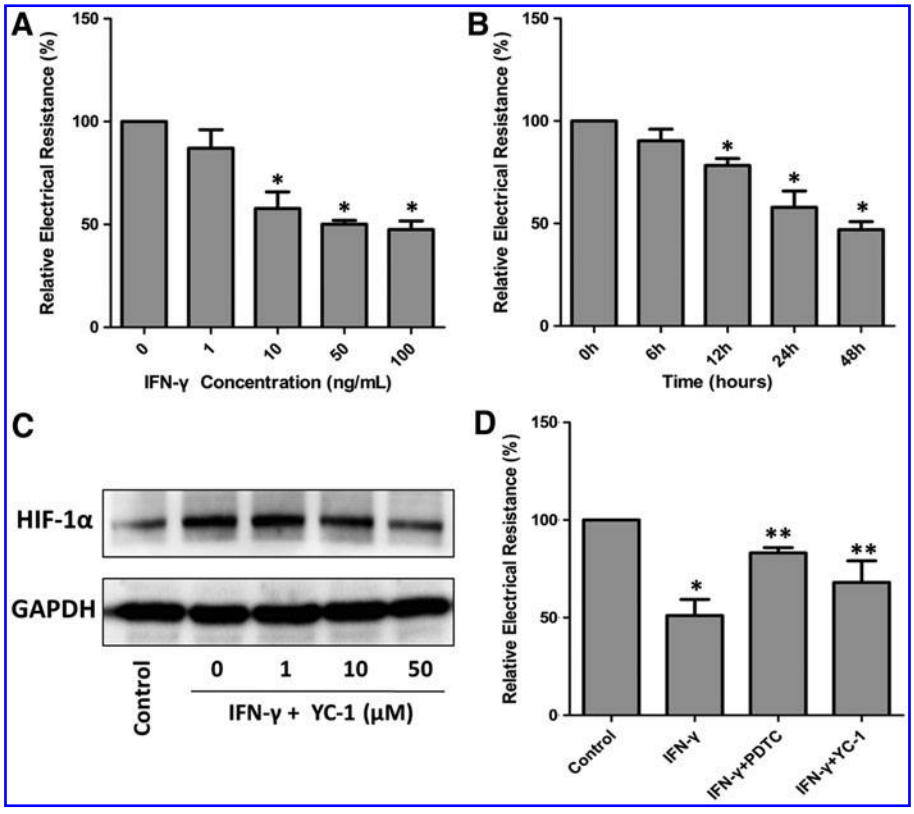

FIG. 4. PDTC and YC-1 prevent IFN-γ-induced intestinal barrier dysfunction. **(A)** T84 cells were treated with different concentration of IFN-γ (0, 1, 10, 50, and 100 ng/mL). IFN-γ produced a dose-dependent decrease in T84 transepithelial electrical resistance (TER) over the 48 h experimental period ($n=4$). *$P<0.05$ versus control. **(B)** 50 ng/mL IFN-γ was added to T84 cells for the indicated time. IFN-γ caused a time-dependent drop in T84 TER ($n=4$). *$P<0.05$ versus control. **(C)** T84 cells were cultured with IFN-γ (50 ng/mL) without or with different doses (0, 1, 10, 50 μM) of YC-1 for 48 h. IFN-γ induced a significant increase in HIF-1α protein expression, whereas YC-1 inhibited the IFN-γ-induced increase in HIF-1α expression at the concentration indicated. **(D)** NF-κB inhibitor PDTC (100 μM) and HIF-1α inhibitor YC-1 (50 μM) significantly prevented the IFN-γ (50 ng/mL) induced drop in T84 TER at 48 h ($n=4$) *$P<0.05$ versus controls. **$P<0.05$ versus IFN-γ-treated T84 cells.

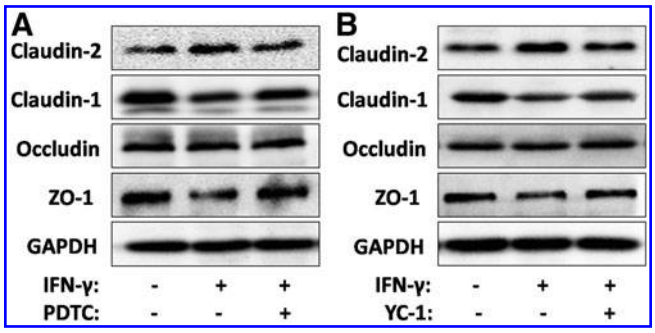

FIG. 5. Effect of IFN-γ on tight junction protein expression in T84 cells. T84 cells were treated with vehicle, IFN-γ (50 ng/mL), or IFN-γ and inhibitors PDTC or YC-1 for 48 h. Protein expression were determined by Western immunoblot. IFN-γ caused a downregulation of ZO-1 and Claudin-1 protein expressions, and IFN-γ also induced an upregulation of Claudin-2 protein expression, which plays an important role in the loss of intestinal barrier function **(A, B)**. While NF-κB inhibitor PDTC (100 μM) **(A)** and HIF-1α inhibitor YC-1 (50 μM) **(B)** significantly attenuated the changes of IFN-γ-induced tight junction proteins.

not shown). As illustrated in Fig. 6, at the site of control T84 monolayers, some tight junctions were bright, spherical accumulations of labeling, representing aggregations of junction proteins. However, T84 cells treated with IFN-γ for 48 h led to a dramatic disruption of tight junctions. Analysis of IFN-γ-treated T84 cells showed that immunostaining pattern for ZO-1 and Claudin-1 was similar but of a decreased intensity, with large portions of the junction appearing dark, which represents discontinuities of tight junctions (Fig. 6). Although no significant changes of Occludin protein expression were found after IFN-γ treatment; however, the morphologic distribution of Occludin became irregular and was interrupted by gaps where tight junction proteins were absent after IFN-γ treatment (Fig. 6 insert image). As well, an internalization of Occludin into the epithelial cell cytoplasm was noted. By contrast, pretreatment with YC-1 (HIF-1α inhibitor) significantly attenuated the morphological changes of tight junction protein ZO-1 and Occludin induced by IFN-γ (Fig. 6). Similarly, NF-κB inhibitor PDTC also attenuated the morphological changes of tight junction proteins caused

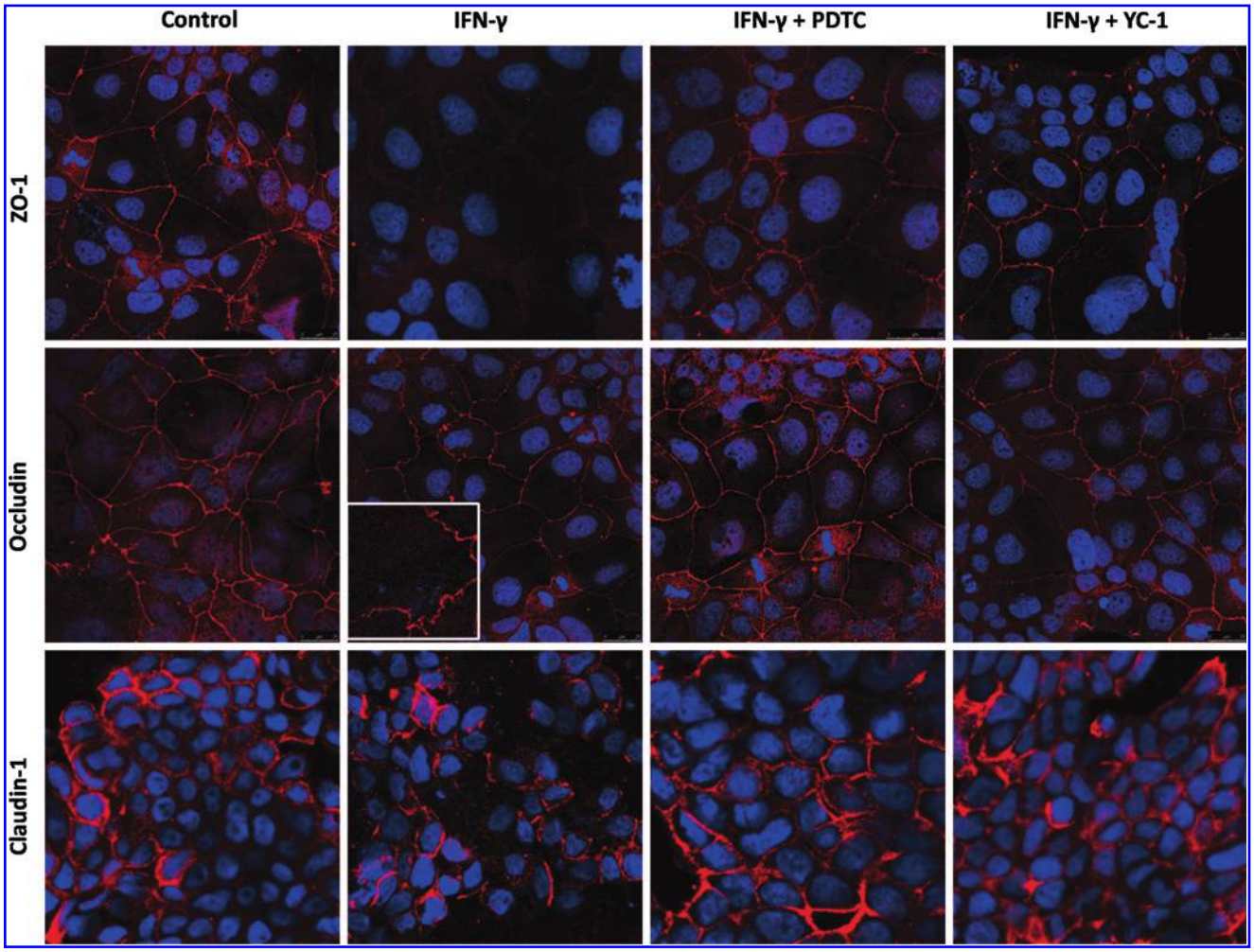

FIG. 6. PDTC and YC-1 attenuated IFN-γ-induced disruption of tight junction proteins. T84 cells were treated with IFN-γ (50 ng/mL) for 48 h. Tight junction proteins ZO-1, Occludin, and Claudin-1 underwent immunofluorescence staining. IFN-γ caused dramatic disruption of tight junctions ZO-1 and Claudin-1. The large proportion of dark represents discontinuities of tight junctions. IFN-γ also caused disruption of the tight junction Occludin. As shown in the insert image, the distribution of Occludin was transformed from regularly smooth into irregular, along with an internalization of the junctional protein. IFN-γ induced morphological distribution of tight junctions were inhibited by NF-κB inhibitor PDTC (100 μM) or HIF-1α inhibitor YC-1 (50 μM). Magnification: ×800.

by IFN-γ (Fig. 6). These results indicate that HIF-1α plays an integral role in IFN-γ-induced intestinal barrier dysfunction.

Discussion

In the present study, we found that IFN-γ-induced epithelial barrier dysfunction by NF-κB/HIF-1α pathway. To determine the roles of NF-κB and HIF-1α in IFN-γ-induced disruption in T84 intestinal epithelial barrier function, NF-κB inhibitor PDTC and HIF-1α inhibitor YC-1 were used. We found that PDTC significantly inhibit IFN-γ-induced activation of NF-κB. YC-1 as well as PDTC attenuates IFN-γ-induced HIF-1α expression and increase of TER. This study also showed that IFN-γ-induced disruption of tight junction proteins were alleviated by PDTC and YC-1. Our study provided new evidence for the mechanisms involved in IFN-γ-induced intestinal barrier dysfunction.

Previous studies have demonstrated that IFN-γ plays pivotal roles in the pathogenesis of intestinal barrier function disruption (Youakim and Ahdieh 1999; Bruewer and others 2005; Willemsen and others 2005). In addition, we have previously demonstrated that IFN-γ evokes epithelial apoptosis and impair intestinal barrier function (Yang and others 2002, 2003). However, the precise mechanism involved in is still unclear. It has been well demonstrated that the NF-κB pathway plays an important role in IFN-γ-induced intestinal barrier dysfunction (Boivin and others 2009). Furthermore, recent studies have shown that some biological effects of IFN-γ are elicited through activation of the NF-kB pathway (Gough and others 2008; Thapa and others 2011; Lin and others 2012). Therefore, in this study, we determined the potential role of the NF-kB pathway in the effects of IFN-γ-induced HIF-1α on T84 intestinal epithelial cells. Our study demonstrates that IFN-γ could activate NF-kB through promoting the nucleus translocation of P65. It is interesting to note that the IFN-γ-induced NF-κB activation demonstrated in this study is delayed in comparison to the induction by other proinflammatory cytokines such as TNF-α or LPS (Boivin and others 2009). It has been shown that TNF-α-induced activation of NF-κB occurs almost immediately following TNF-α treatment (Ma and others 2004; Wang and others 2005). In this regard, the mechanism mediating the IFN-γ activation of NF-κB appears to be quite distinct from that involved in TNF-α or LPS (Zhong and others 2012).

Recent studies have demonstrated that increased HIF-1α expressions are deleterious to intestinal or endothelial barrier integrity (Rosenberger and others 2007; Feinman and others 2010; Kannan and others 2011; Liu and others 2011). Many of the proinflammatory cytokines, such as TNF-α, LPS that induce HIF-1 are known to activate a number of other transcription factors such as NF-κB (Jung and others 2003; Frede and others 2005). As NF-kB is the key transcript factor for HIF-1α expression (Figueroa and others 2002), therefore, we hypothesized that IFN-γ may induce the expression of HIF-1α by NF-kB pathway. We found that IFN-γ could upregulate HIF-1α expression in T84 intestinal epithelial cell lines, and this increased HIF-1α expression could be efficiently inhibited by NF-kB inhibitor PDTC, which suggested that IFN-γ increased HIF-1α expression was by the activation of NF-kB transcription factor. In combination, our data indicated that IFN-γ activates NF-κB pathway, which, in turn, leads to the expression of HIF-1α. This is the first study to show that IFN-γ-upregulated HIF-1α in T84 intestinal epithelial cells is mediated by NF-κB pathway.

YC-1, a multifunctional agent, was first observed to be an activator of platelet guanylate cyclase (Ko and others 1994). More recently, YC-1 was reported to decrease hypoxia-induced HIF-1α accumulation and expression (Chun and others 2001; Sun and others 2007; Li and others 2008; Tsui and others 2012). In the present study, we reveal that YC-1 attenuates intestinal epithelial barrier dysfunction caused by IFN-γ, as evidenced by that YC-1 mitigates TER decrease in intestinal epithelia exposed to simultaneous IFN-γ.YC-1 also prevents IFN-γ-induced disruption of intestinal tight junction, as evidenced by that YC-1 preserves the morphological distribution of tight junction proteins ZO-1 and Occludin in intestinal epithelia challenged with simultaneous IFN-γ. In support of our findings, previous studies have suggested that YC-1 prevents barrier dysfunction and tight junction protein ZO-1 and Occludin disorganization in endothelia (Yeh and others 2007) or intestinal (Liu and others 2011). Additionally, we have found that NF-kB inhibitor PDTC also alleviates TER decrease and distribution of tight junction proteins ZO-1, Occludin, Claudin-1, and Claudin-2 in T84 intestinal epithelial cells caused by IFN-γ. Moreover, the effects of PDTC seem more significantly than YC-1. One most possible reason is that YC-1 inhibits HIF-1α by attenuating the activation of NF-κB. It has been reported YC-1 suppresses HIF-1 accumulation and translational upregulation through inhibition of NF-κB signaling during hypoxia (Sun and others 2007). Another possibility is that the activation of NF-κB may play other deleterious roles in IFN-γ-induced intestinal barrier function disruption by other signal pathways, such as NF-κB/MLCK pathway (Ma and others 2005; Gu and others 2011; He and others 2012). Further studies are needed to fully delineate the intracellular mechanisms that mediate NF-κB modulation of tight junction barrier following IFN-γ stimulation, and the role of NF-κB in the process of YC-1 inhibition HIF-1α. The siRNA of NF-κB and HIF-1α should be used to determine the precise mechanism involved in IFN-γ-induced intestinal epithelial barrier dysfunction in our future studies.

In conclusion, IFN-γ-induced disruption in epithelial barrier function is regulated by NF-κB/HIF-1α pathway. To the best of our knowledge, the current findings, for the first time, demonstrate that IFN-γ-induced activation of NF-κB leads to a sequential activation of HIF-1α pathway and HIF-1α plays an injurious role in IFN-γ-induced epithelial barrier dysfunction. The inhibition of NF-κB and HIF-1α pathways attenuate intestinal epithelial barrier dysfunction caused by proinflammatory cytokine IFN-γ and prevent IFN-γ-induced morphological disruption of tight junction in intestinal epithelia. Taken together, this study provides new insight into the intracellular pathways that mediate the IFN-γ-induced disturbance of intestinal epithelial barrier function.

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Author Disclosure Statement

No competing financial interests exist.

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