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RESEARCH ARTICLE

INTERACTION BETWEEN HUMAN FERRITIN AND OMPA FROM KLEBSIELLA PNEUMONIAEATCC 13883 IN HEP-2 CELLS

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ABSTRACT

Klebsiella pneumonia is a major cause of community- and hospital-acquired bacterial pneumonia. OmpA (KpOmpA) is one of the most abundant virulence factors produced by *K. pneumonia*. This bacterial surface protein is recognized by the host immune system as apathogen-associated molecular pattern (PAMP). We used a yeast two-hybrid assay to examine novel KpOmpA-human protein binding interactions. The major intracellular iron-storage protein, ferritin, was one of the four proteins identified. We hypothesized that the virulence mediated by KpOmpA could be at least partly due to a direct, inflammation-associated and cell cycle-arresting binding interaction between KpOmpA and the host ferritin. We confirmed the direct binding between KpOmpA and ferritin in HEp-2 cells. We also found that KpOmpAco-localized with ferritin within the perinuclear region, and enveloped the entire ferritin surface. KpOmpA treatment resulted in G2/M phase cell cycle arrest. Transcription and expression of pro-inflammatory cytokines (IL-1β, IL-6, IL-8, and TNF-α) also increased. This study revealed a novel interaction between KpOmpA and human ferritin that may have a significant role in *K. pneumoniae* virulence. The results indicated that KpOmpAis a potential therapeutic target for the treatment of *K. pneumoniae* infection.

INTRODUCTION

Klebsiella pneumoniae is an opportunistic pathogen and the most common gram-negative bacterial agent isolated from community- and hospital-acquired (nosocomial) pneumonia patients. Immunocompromised individuals are particularly vulnerable to *K. pneumoniae* infection (Sahly & Podschun, 1997). Multidrug-resistant *K. pneumoniae*can be found worldwide and is spreading rapidly to new geographic areas (Chen *et al*, 2012; Giamarellou, 2005). Antibiotic resistance complicates treatment and limits therapeutic options inclinical settings; new treatment targets and approaches must be identified. Some bacterial molecules (e.g., siderophores, lipopolysaccharide, adhesins, and capsule polysaccharides (CPSs)) are well-known *K. pneumoniae* virulence factors and potential treatment targets. The role of CPSs has been a major focus of *Klebsiella* virulence research (Cano *et al*, 2009).

The bacterial outer membrane vesicles (OMVs) contribute to the virulence of gram-negative bacteria. The outer membrane and its proteins are transported via these OMVs (Mashburn-Warren *et al*, 2008), which fuse with host cell membranes and secrete their components into the host cell cytosol. Among the *K. pneumonia*-produced proteins transported by OMVs*in vitro* and *in vivo,* outer membrane protein A (OmpA) is one of the most abundant and virulent (Lee *et al*, 2012). OmpAis highly conserved within the *Enterobacteriaceae* family. OmpA is targeted by, but also evades, the immune system. The proteinhas four extracellular loops (pathogen-associated molecular patterns (PAMPs)), which are recognized by the immune system (Jeannin *et al*, 2002). After OmpA binds to the host cell surface and moves to the cell interior, it activates macrophages and professional antigen-presenting cells (e.g., dendritic cells) (Pichavant *et al*, 2003).

We used a yeast two-hybrid screen to identify novel *K. pneumonia* OmpA (KpOmpA)-human ferritin interactions. We also investigated host cell cycle variation and inflammatory responses toKpOmpA. Our results are the first to suggest that

KpOmpA causes cell cycle arrest and pro-inflammatory cytokine induction.

MATERIALS AND METHODS

Cell culture and transfection

Human epithelial type 2 (HEp-2) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum and 1000U penicillin-streptomycin (Gibco BRL). The cells were maintained in DMEM supplemented with 10% (v/v) and antibiotics; they were incubated in a humidified atmosphere (5% CO_2) at 37^{o}C.

Plasmid constructs

Klebsiella pneumoniae genomic DNA was used as template. It was amplified using the forward and reverse primers5′-CCAAGAATTCATGAAAAAGACAGCTATCGC-3′and 5′-AACCGTCGACAAGCCGCCGGCTGAGTTAC-3′, respectively. The PCR product was cloned into the pET28a and pGBKT7 vectors (Clontech, Palo Alto, CA, USA). The PCR products were digested using EcoRI and SalI, and cloned into pET28a (recombinant KpOmpA) plasmid vectors. DNA sequencing was used to confirm all cloned constructs. His-gagged recombinant OmpA protein expressed in *Escherichia coli* BL21 (DE3) cells was purified using Ni-agarose beads, according to the manufacturer's instructions (Amersham Biosciences, CA, USA). The purified proteins were used for the pull-down assays.

Yeast two-hybrid assay

The Matchmaker Gold Yeast Two-Hybrid System (protocol PT4084-1; Clontech, Palo Alto, CA, USA) was used to screen a human liver cDNA library for proteins that interacted with OmpA. The bait plasmid (OmpA-pGBKT7) was transformed into the AH109 yeast strain; approximately 2×10^3 transformants were screened. The transformants were assayed for MEL1 activation using selection on high-stringency medium plates (SD/-Ade/-His/-Leu/-Trp/X-α-gal). The Zymoprep kit (Clontech, Palo Alto, CA, USA) was used to recover the prey plasmids from positive yeast clones; the plasmids were then retransformed into DH5α cells. The transformants that contained activation domain plasmids were selected using Luria-Bertani plates with 100μg/ml ampicillin. DNA sequencing was used to identify the plasmid inserts.

Immunoblotting

The pulled-down or immunoprecipitated KpOmpA (or ferritin) was resolved using a 12% SDS-PAGE gel. The product was transferred to a PVDF membrane (Invitrogen, Carlsbad, CA), which was then incubated in blocking buffer (5% dried skimmed milk in phosphate-buffered saline [PBS] and 0.05% Tween-20) and probed using specific antibodies followed by a horseradish peroxidase-conjugated secondary antibody. A commercial western blotting detection system (Pierce, Rockford, IL, USA) was used to detect the immune complexes.

Immunoprecipitation

The HEp-2 cells were analyzed 48 hours after a 20 μg/ml treatment with KpOmpA. The cells were rinsed with ice-cold phosphate-buffered saline and resuspended in 1 ml RIPA buffer (10mMTris-HCl pH7.4, 1mM EDTA, 5mMDTT, 100mM NaCl, 1.0% Triton X-100, 60mM n-octylglucoside, 1mMvanadate, 100μMmolybdate, 20mM sodium fluoride, and protease inhibitor cocktail [1 tablet per 10ml extraction buffer]).The pre-cleaned lysate was incubated (1 hour, 4^{o}C) with His-OmpAtagor ferritin antibody. Protein A-Sepharose beads (Pharmacia Co., NJ, USA) were used to collect the resulting immune complexes. The solution was then centrifuged to capture the immune complexes. After centrifugation, the product was washed three times in RIPA buffer and solubilized using a 2x sample buffer before loading onto a 12% SDS-PAGE gel.

OmpA pull-down assay

The His-OmpA (KpOmpA) fusion proteins were purified on a His-binding resin (Novagen, Madison, WI); 1 μg His-tagged recombinant OmpA was then added to whole cell lysates of HEp-2 cells. End-over-end rotation during a 2-hour incubation (4°C) allowed for association between the KpOmpA and ferritin. The associated protein complexes were collected from His-binding resin slurry, and thoroughly washed. The samples were resuspended in 2x Laemmli sample buffer and analyzed using a 12% SDS-PAGE gel and a western blot with ferritin antibodies.

Confocal microscopy

The HEp-2 cells were seeded overnight (70% confluence) onto culture slides coated with human fibronectin (SPL, Korea). The cells were treated with the His-OmpA (KpOmpA) the following day and incubated for 48 hours. They were then washed several three times with ice-cold PBS and fixed in 2% paraformaldehyde for 10 minutes. The fixed cells were permeabilized using 0.1% Triton X-100 for 10 minutes and blocked for 2 hours in PBS containing 5% bovine serum albumin (BSA; Aurion, The Netherlands) and 0.1% Tween. The slides were washed three times using 0.01% PBS-Tween after an overnight incubation (4°C) with a monoclonal (rabbit) antibody against His-OmpA (1:100, in 5% BSA-PBS; Bio-Protocol, Palo Alto, CA, USA). Alexa Fluor 568 or 488-conjugated donkey anti-rabbit (1:200, in 5% BSA-PBS; Molecular Probes, Inc., Eugene, OR, USA) was used as a secondary antibody. The confocal microscopy analysis was performed using an LSM710 (Zeiss, Germany) at the Center for Research Instruments and Experimental Facilities of Chungbuk National University. The protein co-localization was detected, and the confocal microscopic images were scanned, using the Profile feature in the ZEN software program provided by the manufacturer.

FACS analysis

The cell cycle of the host cells was determined using flow cytometry. HEp-2 cells were maintained in 12-well culture dishes. The cell cycle was synchronized viaa 12-hour serum starvation. After reactivation, the HEp-2 cells were exposed to 20μg His-tagged recombinant OmpA for 24 hours. The cells were detached from the wells using Trypsin-EDTA, washed with PBS, fixed using 70% ethanol, and stained with propidium iodide. Sample fluorescence was measured using an FACSCalibur flow cytometer (BD Biosciences, New Jersey, USA) and analyzed using FlowJo 7.5.2 software (TreeStar, Ashland, OR, USA) at the Core Facility of Chungbuk National University.

Reverse transcription polymerase chain reaction (RT-PCR)

HEp-2 cells were exposed to 20μg His-tagged recombinant OmpA for 24 hours. The total RNA was prepared using Trizol.

Reverse transcription was used to synthesize first-strand cDNA. Using the cDNA as the template, RT-PCR was performed using an Eppendorf Mastercycler PCR system (Eppendorf, NY, USA). The primers used for the RT-PCR are presented in Table 1. The quantitative RT-PCR data for each sample were normalized to the internal control (GAPDH), and the fold changes for the treated samples, relative to the control samples, were calculated.

Statistical analysis

The numerical results are presented as mean ± standard deviation values from at least four independent experiments. Each experiment was performed three times. The Student's t-test was used to analyze differences between the control and experimental groups for the measured variables. A value of * $P < 0.05$ or ** $P < 0.01$ indicated a statistically significant difference.

RESULTS

Identification of OmpA-human protein interactions

A yeast two-hybrid system was used to screen a human liver cDNA library; *K. pneumoniae* OmpA was used as bait. We identified 23 positive clones representing nine putative proteins (Figure 1). Four of these putative proteins were designated as ferritin, glucuronidase, aldolase, and selenoprotein; the other five proteins were genomic contigs or scaffolds (Table 2).

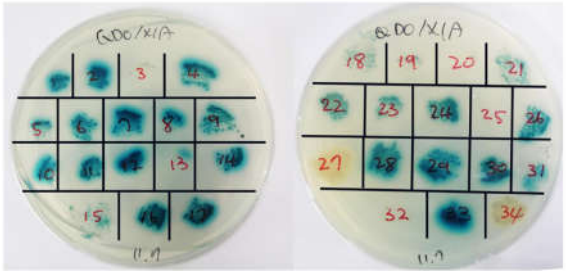

Figure 1 Screening of KpOmpA-interacting human proteins using the yeast two-hybrid system. An OmpA-pGBKT7 construct was transformed into yeast AH109 cells as bait. The prey was a human liver cDNA library. Leucine, adenine, tryptophan, aureobasidin A, and histidine were used to select positive colonies. A total of 23 positive clones (blue) were selected. Nine proteins were identified. Four of these were designated, and five were genomic contigs or scaffolds, as revealed by DNA sequencing.

Endogenous ferritin interacts with KpOmpA

We verified the direct interaction between KpOmpA and human ferritin using immunoprecipitation of lysates from HEp-2 cells treated with KpOmpA (20μg/ml). The KpOmpA was present in immunoprecipitates from cell lysates immobilized with anti-FTH and anti-FTL antibodies, but not in immunoprecipitates containing only immunobeads (Figure 2A). The results of a reciprocal immunoprecipitation experiment indicated that OmpA and ferritin directly interact; KpOmpA was present in cell lysates immobilized with FTH antibody, but not in the immunobead control (Figure 2B).A His pull-down assay revealed the physical interaction between KpOmpA and ferritin. Ferritin was pulled down with KpOmpA, but not with His-resin alone (Figure 2C). This result indicated that there was physical contact between the two proteins.

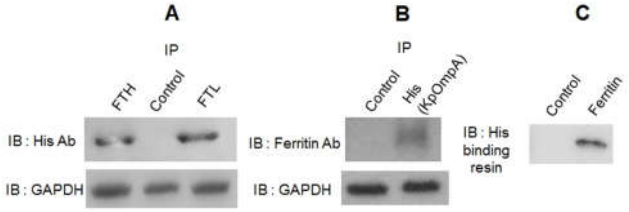

Figure 2 Confirmation of OmpA-ferritin interaction using immunoprecipitation (IP) and His pull-down assays. (A)SDS-PAGE and western blot of IP of lysates from His-KpOmpA(20μg/ml) treated HEp-2 cells. The western blot was stained using anti-FTH and anti-FTL antibodies. (B) Using an anti-His antibody, anti-ferritin immunoprecipitated complexes were subjected to immunoblotting. (C) His binding resin-KpOmpA pulls down HEp-2 ferritin. Total soluble HEp-2 cellular protein (lysate) was loaded in the first lane as a reference for ferritin. The left lane (control) was loaded with the His-resin elute after purification and washing. The results represent three separate experiments.

KpOmpA and ferritin co-localize in the perinuclear region

We used confocal microscopy to examine whether human ferritin associated with KpOmpA in HEp-2 cells. We analyzed the immunofluorescence of KpOmpA (20μg/ml) treated in HEp-2 cells. Endogenous ferritin and KpOmpA co-localized in the perinuclear region (Figure 3).

Cell cyclearrest at the G2/M phaseinduced by KpOmpA

Cells exposed to oxidative stress typically arrest at the G2/M phase of the cell cycle (Li *et al*, 2009). We examined whether KpOmpA could induce G2/M arrest, which would suggest that KpOmpA induces oxidative stress in host cells. Flow cytometry analysis revealed that the percentage of HEp-2 cells, arrested at theG2/M phase, increased 3.1-fold after KpOmpA

Table 1 The sequences for the primers used for RT-PCR to quantify expression of pro-inflammatory genes

Target	Sense Primer	Antisense primer	Expected size (bps)
GAPDH	5' - TGGAAGGACTCATGACCACA - 3'	5' - TTCAGCTCAGGGATGACCTT -3'	163
IL-1β	5' - GGGCCTCAAGGAAAAGAATC -3'	5' - TTCTGCTTGAGAGGTGCTGA - 3'	205
IL-6	5' - TGGTCTTTTGGAGTTTGAGGTA - 3'	5' - AGGTTTCTGACCAGAAGAAGGA - 3'	346
IL-8	5' - GTGCAGTTTTGCCAAGGAGT - 3'	5' - CTCTGCACCCAGTTTTCCTT - 3'	196
TNF-α	5' - GTGGCAGGCGCCACCACGCTCTTC - 3'	5' - GAAGAGGACCTGGGAGTAGATGAG - 3'	300

Table 2 Proteins identified from *Klebsiella pneumoniae* OmpA-human liver cell yeast two-hybridization screening

Access No.	Description	Max score	Total score	Query coverage	Max ident
NM_000181.3	Homo sapiens glucuronidase, beta (GUSB)	110	110	100%	100%
NM_000146.3	Homo sapiens ferritin, light polypeptide (FTL)	1379	1379	99%	99%
NM_000035.3	Homo sapiens aldolase B, fructose-bisphosphate (ALDOB)	1752	1752	99%	96%
NM_001093726.1	Homo sapiens selenoprotein P, plasma, 1 (SEPP1), transcript variant 3	927	927	96%	98%
NM_002032.2	Homo sapiens ferritin, heavy polypeptide 1 (FTH1)	148	148	58%	68%
NM_005165.2	Homo sapiens aldolase C, fructose-bisphosphate (ALDOC)	424	424	53%	77%

This result suggested that KpOmpA induced host cell oxidative stress.

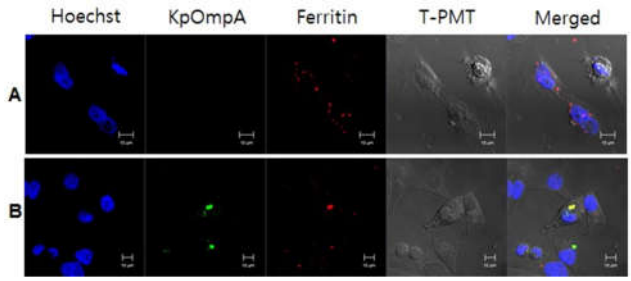

Figure 3 Interaction between ferritin and KpOmpA and the subcellular localization of ferritin and KpOmpA. (A) Endogenous ferritin (red). (B) Treated 20μg/ml His-KpOmpA (green). Endogenous ferritin in HEp-2 cells is shown in confocal fluorescence micrographs. This protein was visualized using immunofluorescence in fixed and permeabilized cells. Monoclonal or polyclonal antibodies against human ferritin or His-KpOmpA, and Alexa Fluor 568-conjugated donkey anti-rabbit IgG or Alexa Fluor 488-conjugated mouse anti-rabbit IgG, were used. The yellow pattern (i.e., red combined with green) indicates protein co-localization in the perinuclear region (blue in the nucleus). Hoechst staining (blue color) was used to visualize the cell's nuclear region. Scale bar, 10μm.

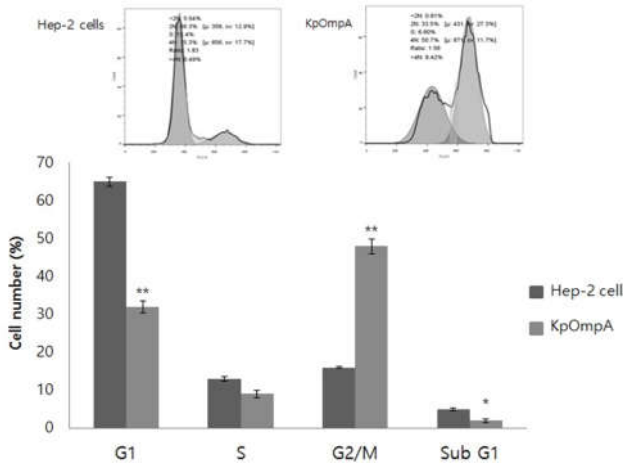

Figure 4 G2/M phase OmpA-induced cell cycle arrest. The cells were synchronized using serum starvation. Cells were then reactivatedand treated with His-KpOmpA (20μg/ml). After fixation and staining, the cell cycle was determined using FACSCalibur. The histogram patterns represent three separate experiments, and the results presented are the mean ± standard deviation (S.D.)values of three separate experiments. *p< 0.05, **p< 0.01.

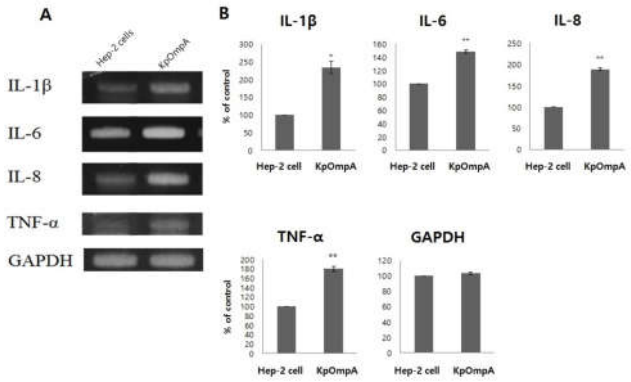

Figure 5 Pro-inflammatory cytokines are stimulated by KpOmpA in HEp-2 cells. (A) HEp-2 cells were treated using His-KpOmpA (20μg/ml), followed by RNA isolation and targeting of IL-1β, IL-6, IL-8, and TNF-α for RT-PCR. A representative agarose gel electrophoresis product of RT-PCR-amplified cDNAs, corresponding to pro-inflammatory cytokines, is presented. (B) Densitometry analysis of the bands revealed that KpOmpA treatment up regulated the transcription ofIL-1β, IL-6, IL-8, and TNF-α. The results presented are the mean ± standard deviation (S.D.)values of three experiments. *p< 0.05, **p< 0.01.

KpOmpA evokes a pro-inflammatory cytokine response in HEp-2 cells

Because *K. pneumoniae* OMVs have been associated with pro-inflammatory cytokine induction (Lee *et al*, 2012), and KpOmpA is transported by OMV's, we examined whether KpOmpA induced a host cell pro-inflammatory cytokine response. The mRNA levels of the pro-inflammatory cytokines IL-1β, IL-6, IL-8, and TNF-α increased 2.5-fold, 1.5-fold, 1.9-fold, and 1.9-fold, respectively, in KpOmpA-treated cells compared with non-treated cells (Figure 5).

DISCUSSION

This study revealed novel interactions between KpOmpA and human ferritin. Ferritin is the major intracellular protein involved in iron storage. It occurs in almost every living systemand has a highly-conserved structure (Torti & Torti, 2002). Ferritin controls intracellular iron. It can sequester up to 4500 iron atoms and store excess iron. Ferritin is a 24-subunit protein composed of two subunit types (i.e., the heavy chain and the light chain). Heavy chain have ferroxidase activity, which accelerates Fe(II) oxidationoxidizes iron to its Fe(III) form; the heavy chain also participates in rapid iron detoxification. The light chain facilitates iron nucleation, mineralization, and long-term iron storage (Harrison & Arosio, 1996). Recent studies have revealed that the light chain promotes cell growth, and the heavy chain regulates and buffers cellular iron availability (Cozzi *et al*, 2004). The yeast two-hybrid screening results indicated that KpOmpA bound to ferritin, glucuronidase, aldolase, and selenoprotein in yeast two-hybrid screening. Of the four proteins identified, ferritin seemed the most likely to be targeted directly by *K. pneumoniae* during an infection because it can sequester iron. Iron sequestration is essential for bacterial cell growth in the host (Harrison & Arosio, 1996). Bacterial binding of ferritin could also hinder the host cell due to ferritin's ability to promote cell growth (Cozzi *et al*, 2004).

The immunoprecipitation and pull-down assay results indicated that KpOmpA interacted directly with ferritin (Figures 1, 2). When cells were treated with KpOmpA, the KpOmpA enveloped the entire surface of the ferritin in the HEp-2 cells (Figure 3). OmpA internalization evokes an immune response (Soulas *et al*, 2000). Our results indicate that His-tagged KpOmpA directly infiltrated HEp-2 cells without assistance from an artificial vector.

Apart from regulating oxidative stress control at theprotein level, our results indicate another effect of oxidative stress. The HEp-2 cell cycle was arrested at the G2/M phase at a rate that was 3.1-fold higher than the rate in the control groups (Figure 4). This cell cycle arrest could have been caused by cyclin B1 and cyclin-dependent kinase1 (cdc2) down regulation by KpOmpA (data not shown). Mitosis entry is mediated by cyclin B1and cdc2. Precise regulation of cyclin B1/cdc2 complexes guarantees entry into mitosis. Phosphorylation of cdc2 controls entry into the M phase (Smits & Medema, 2001). Oxidative stress prevents cell proliferation by downregulating cyclin B1, therefore inducing G2/M cell cycle arrest (Li *et al*, 2009). H_2O_2 decreases cyclin B1 expression via methylation of the transcription factor Sp1. Histone deacetylase 1 (HDAC1) is attracted to the cyclin B1 promoter via methylation of Sp1; histone H3 methylation follows cyclin B1 downregulation (Chuang *et al*, 2011). We suggest that this oxidative stress can arrest the cell cycle via

downregulation of cyclin B1 and cyclin-dependent kinase 1 (cdc2), because oxidative stress-mediated cyclin B1 down regulation induces G2/M cell cycle arrest (Li *et al*, 2009). Taken together, our results indicate that oxidative radical accumulation, induced by KpOmpA, might down regulate cyclin B1/cdc2 and cause HEp-2 G2/M cell cycle arrest.

Klebsiellapneumoniae causes pneumonia, which is a representative inflammatory disease. KpOMVs induce pro-inflammatory cytokines (Lee *et al*, 2012). KpOmpA up regulates IL-1β, IL-8, IL-10, IL-12, and TNF-α production by binding to human macrophages (Soulas *et al*, 2000). However, there is another mechanism for pro-inflammatory cytokine induction. Increased reactive oxygen species (ROS) levels can activate NF-κB; NF-κB has a significant role in pro-inflammatory gene expression regulation. NF-κB mediates the synthesis of several cytokines (e.g., TNF-α, IL-1β, IL-6, and IL-8) (Tak & Firestein, 2001). H_2O_2 activates NF-κB via Syk protein-tyrosine kinase-mediated phosphorylation of IκBα (Takada *et al*, 2003). We found that KpOmpA activated the transcription of IL-1β, IL-6, IL-8, and TNF-α (Figure 5). It also increased the secretion of IL-6 and IL-8 (Figure 5) by HEp-2 cells. IL-1β is an important initiator of acute-phase inflammatory responses to infection (Dinarello, 1998). IL-6 affects B cell maturation, antigen-specific immune responses, and hepatic synthesis of acute phase proteins (Cotler *et al*, 2001). IL-8 controls neutrophil and lymphocyte infiltration and protects hosts challenged with *K. pneumoniae* (Standiford *et al*, 1996). The pro-inflammatory cytokine TNF-α participates in apoptotic cascade activation (Motani *et al*, 2010). In summary, KpOmpA induced oxidative stress and activated NF-κB, which is a key factor in pro-inflammatory response mediation.

This study revealed a novel interaction between KpOmpA and human ferritin. The interaction attenuated ferritin function, which causes accumulation of oxidative radicals. The increase in ROS arrested the cell cycle at the G2/M phase and evoked a pro-inflammatory cytokine response. This study is the first to reveal this KpOmpA-ferritin interaction. The effects of this interaction in HEp-2 cells may provide a basis for future studies on KpOmpA-ferritin mediated *K. pneumoniae* infection and the virulence of KpOmpA.

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