Biochemical and Biophysical Research Communications 416 (2011) 153–158

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Rickettsia conorii infection stimulates the expression of ISG15 and ISG15 protease UBP43 in human microvascular endothelial cells

Punsiri M. Colonne [a,b], Abha Sahni [c], Sanjeev K. Sahni [c,d,*]

[a] Department of Pathology and Laboratory Medicine, University of Rochester School of Medicine and Dentistry, Rochester, NY, United States
[b] Department of Microbiology and Immunology, University of Rochester School of Medicine and Dentistry, Rochester, NY, United States
[c] Department of Pathology, University of Texas Medical Branch, Galveston, TX, United States
[d] Institute for Human Infections and Immunity, University of Texas Medical Branch, Galveston, TX, United States

ARTICLE INFO

Article history:
Received 28 October 2011
Available online 11 November 2011

Keywords:
Endothelial cells
Interferon
ISG15
Rickettsia conorii
UBP43

ABSTRACT

Rickettsia conorii, an obligate intracellular bacterium and the causative agent of Mediterranean spotted fever, preferentially infects microvascular endothelial cells of the mammalian hosts leading to onset of innate immune responses, characterized by the activation of intracellular signaling mechanisms, release of pro-inflammatory cytokines and chemokines, and killing of intracellular rickettsiae. Our recent studies have shown that interferon (IFN)-β, a cytokine traditionally considered to be involved in antiviral immunity, plays an important role in the autocrine/paracrine regulation of host defense mechanisms and control of *R. conorii* growth in the host endothelial cells. Here, we show that *R. conorii* infection induces the expression of ISG15 (an interferon-stimulated gene coding a protein of 17 kD) and UBP43 (an ISG15-specific protease) at the levels of mRNA and protein and report the evidence of ISGylation of as yet unidentified target proteins in cultured human microvascular endothelium. Infection-induced expression of ISG15 and UBP43 requires intracellular replication of rickettsiae and production of IFN-β, because treatment with tetracycline and presence of an antibody capable of neutralizing IFN-β activity resulted in near complete attenuation of both responses. Inhibition of *R. conorii*-induced ISG15 by RNA interference results in significant increase in the extent of rickettsial replication, whereas UBP43 knockdown yields a reciprocal inhibitory effect. In tandem, these results demonstrate the stimulation of interferon-β-mediated innate immune mechanisms capable of perturbing the growth and replication of pathogenic rickettsiae and provide first evidence for ISG15-mediated post-translational modification of host cellular proteins during infection with an intracellular bacterium.

1. Introduction

Rickettsia conorii is an obligate intracellular Gram-negative bacterium, which causes Mediterranean spotted fever (MSF), a serious and occasionally fatal exanthematic disease typically characterized by fever, maculopapular rash, and 'tache-noire' at the site of tick bite [1]. The past few years have witnessed increased recognition and spread of pathogenic *Rickettsia* species, including *R. conorii*, throughout the world and identification of new and previously unsuspected vectors capable of transmitting the disease to humans [2,3]. Additionally, more recent descriptions of MSF patients have also attributed previously underappreciated complications such as acute myocarditis [4], acute pancreatitis [5], and persistent encephalitis [6] as compounding factors of the progression and outcome of human *R. conorii* infections.

Because microvascular endothelium lining of blood vessels is the primary target of a majority of pathogenic rickettsiae, 'vasculitis' as a result of inflammation, dysfunction, and damage to the vasculature represents a hallmark feature of rickettsial pathogenesis in their mammalian hosts. As expected, endothelial cells infected with spotted fever group rickettsiae launch a combination of responses, which are governed by intracellular signaling pathways leading to the activation of transcriptional control and pathogen clearance mechanisms in the host [7,8]. In this regard, our recent findings demonstrate that *R. conorii* infection of human microvascular endothelial cells *in vitro* induces the expression and secretion of interferon-β (IFN-β: a type I IFN), leading to the autocrine/paracrine stimulation of Janus Kinase-Signal Transducer and Activator of Transcription (JAK-STAT) signaling [9].

Known to be induced by type I IFNs, the human *ISG15* gene encodes for the interferon-stimulated protein of 17 kDa, a ubiquitin-like modifier protein, which can also function as a cytokine [10]. Interestingly, ISG15 within the cells can be detected as both free, unconjugated protein as well as in covalent complexes with other cellular proteins via a process termed 'ISGylation'. Since

* Corresponding author at: Department of Pathology, MMNP 5.210, Mail route 0428, University of Texas Medical Branch, 301 University Boulevard, Galveston, TX 77555, United States. Fax: +1 409 747 2400.
E-mail address: sksahni@utmb.edu (S.K. Sahni).

0006-291X/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2011.11.015

ISG15 expression and activity is under tight control of specific signaling mechanisms governing innate immunity and ISGylation is known to modulate JAK-STAT signal transduction pathway, we hypothesized an important role for ISG15 in host cell responses to pathogenic rickettsiae. In the present study, we report on the expression of ISG15 and UBP43 (the only known ISG15-specific deconjugating protease that removes ISG15 from its conjugates by deISGylation) in microvascular endothelial cells infected with *R. conorii*. Our data clearly suggest increased expression of ISG15 and UBP43 and implicate a role for both of these proteins in host defense via pronounced effects on intracellular replication of *R. conorii*.

2. Materials and methods

2.1. Cell culture, R. conorii infection, and tetracycline treatment

Human dermal microvascular endothelial cells (HMECs) were grown in MCDB 131 medium (Invitrogen) containing 10% FBS (Aleken Biologicals), 10 ng/ml Epidermal growth factor (Becton–Dickinson), 1 μg/ml Hydrocortisone (Sigma), and 10 mM L-glutamine (Invitrogen). *R. conorii* (Malish 7 strain) was propagated in Vero cells, purified by density gradient centrifugation, and titered by plaque formation assay as described previously [11]. HMECs were infected with *R. conorii* for 3 h, at which time initial inoculum of medium supplemented with the bacteria was removed and replaced with culture medium only. Endothelial cells infected with approximately 6×10^3 pfu/cm^2 of *R. conorii* were subjected to the isolation of total RNA and preparation of protein lysates following established laboratory protocols [9,12]. For the analysis of intracellular *R. conorii* replication by quantitative RT-PCR, HMECS were infected with approximately 6×10^2 pfu for every cm^2 of culture surface area. To inhibit intracellular growth of *R. conorii*, tetracycline (Sigma) at a final concentration of 20 μg/ml was introduced into the culture medium at 3 h post-infection [9,11].

2.2. IFN-β treatment of HMECs and neutralization of IFN-β activity

To investigate ISG15 and UBP43 expression, HMECs were treated with 10 ng/ml of recombinant human IFN-β (PBL Interferon Source). For neutralization of IFN-β, an antibody against human IFN-β (R&D Systems) was added to the culture medium after *R. conorii* infection at a final concentration of 10 μg/ml.

2.3. Quantitation of R. conorii by real-time PCR

Either uninfected or *R. conorii*-infected HMECs from the wells of 6-well culture plates were scraped into the medium and collected by centrifugation at 10,000*g* for 30 min. Total DNA (host and rickettsial) was extracted using DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions and quantified by a Nanodrop spectrophotometer (ND-1000, Thermo Scientific). The PCR for rickettsial outer membrane protein A (ompA) was performed using primer pair RR190.547F and RR190.701R with established specificity for spotted fever group rickettsiae [9,13]. The copy number of *R. conorii* DNA in each well was normalized to the total DNA.

2.4. siRNA for ISG15 and UBP43

ON-TARGETplus smart pools of siRNA for ISG15 and UBP43 along with control non-interfering siRNA were purchased from Thermo Scientific. HMECs at or near 80% confluence were transfected with 100 nM siRNA using Lipofectamine 2000™ (Invitrogen) following the manufacturer's recommendations. After 6 h, cells were placed in fresh culture medium and allowed to recover at 37 °C for 12 h prior to *R. conorii* infection.

2.5. Western blotting

Cells were washed with PBS and suspended in a protein lysis buffer containing 2% w/v sodium dodecyl sulfate (SDS). The samples were separated on a 10% polyacrylamide-SDS gel under denaturing conditions, followed by wet gel transfer to a nitrocellulose membrane in a blotting apparatus at 100 V for 90 min. Primary antibodies against ISG15 and UBP43 were purchased from Cell Signaling Technology. An antibody against human α-tubulin from Accurate Chemical & Scientific Corporation was used to control for any variations in loading of samples in different lanes. For detection, IgG-HRP secondary antibodies compatible with the primary antibodies and Western Lightning enhanced chemiluminescence reagent (PerkinElmer) were used.

2.6. Gene expression analysis by quantitative real-time PCR

Total RNA isolated using TRI™ Reagent (Molecular Research Center, Inc.) was purified further using a qPCR-grade RNA purification kit (Qiagen). Conversion of RNA to corresponding cDNA was carried out using a RT^2 First Strand Kit (Qiagen). Primers for human ISG15, UBP43 and GAPDH, and SYBR Green Master mix were obtained from Qiagen. Quantitative PCR reactions were performed according to the manufacturer's instructions in a MyiQ cycler (Bio-Rad). Gene expression was normalized to GAPDH and relative expression was calculated by $^{\Delta\Delta}$Ct method.

2.7. Densitometric and statistical analysis

Blots were scanned in the grayscale mode at a resolution setting of 600 dpi and band intensities were calculated with an image analysis program namely ImageJ, version 1.42. These values were then normalized to the housekeeping protein α-tubulin and the protein expression levels were determined relative to the controls. Statistical significance between the test and control groups was evaluated by Students *t*-test and differences were considered to be statistically significant at a p value of equal to or less than 0.05.

3. Results

3.1. R. conorii infection of HMECs induces the expression of mRNAs encoding ISG15 and UBP43

Microvascular endothelium lining of small and medium-sized blood vessels represents the primary target of infection and an important player in host defense against pathogenic rickettsiae. Therefore, we initially determined the expression of ISG15 and ISG15-specific isopeptidase UBP43 during *R. conorii* infection of HMECs. Compared to the relatively low basal expression in uninfected cells, the mRNA expression of both ISG15 (Fig. 1A) and UBP43 (Fig. 1B) was significantly higher in *R. conorii*-infected HMECs at 48 and 72 h post-infection. The intensity of changes in ISG15 mRNA expression was, however, more pronounced than that of UBP43.

3.2. R. conorii infection and IFN-β treatment of HMECs stimulates the expression of free ISG15 and both isoforms of UBP43: evidence for ISGylation of target proteins

Our follow up experiments to evaluate the steady-state levels of protein products by Western blot analysis further revealed that infection-induced changes in the transcript levels were paralleled