African Journal of Microbiology Research Vol. 5(16), pp. 2245-2248, 18 August, 2011
Available online http://www.academicjournals.org/ajmr
DOI: 10.5897/AJMR11.549
ISSN 1996-0808 ©2011 Academic Journals

Full Length Research Paper

mRNA expression of iron metabolism relation genes in macrophages by infection with *Salmonella typhimurium*

Pan Xin

Department of Microbiology, Second Military Medical University, Shanghai 200433, China. E-mail: xinpanpx@163.com. Tel: 86-21-81870998 or 13524211027. Fax: 86-21-81870998.

Accepted 30 July, 2011

A total number of 13 iron metabolism relation genes expression profiles of RAW264.7 murine macrophages infected or uninfected with *Salmonella typhimurium* were tested by real-time polymerase chain reaction (RT-PCR) to evaluate the metabolism of iron in host-pathogen interplay. The living wild-type *S. typhimurium* induces expression of the transferrin receptor (Tfr1) in host cell macrophages, which results in a sustained increase of the labile iron pool inside the host cell after 1 or 24 h infection. Gene expression analysis showed that wild-type *S. typhimurium* drives an active iron acquisition program with induction of ferrireductase (Steap3), iron membrane transporter Dmt1, and iron regulatory proteins (Irp1 and Irp2), while not too much iron efflux changing through ferriportin (Fpn1). The *spiA*⁻ *Salmonella* mutant strain used in our studies also caused an increase in Tfr1 at 1 or 24 h, but leading to decrease in Fpn1 at 24 h as compared with 1 h. The assessment of the labile iron pool after infection with *spiA*⁻ *Salmonella* after 24 h shows an increase. The same of these two phenotypes allowed iron overload in macrophage and became one of the reasons for *Salmonella* survival inside the macrophage.

Key words: Macrophage, *Salmonella,* iron metabolism.

INTRODUCTION

Iron is a critical determinant in host-pathogen interaction. It is required for full virulence expression by a wide variety of intracellular bacteria. Deprivation of iron in-vivo and in-vitro severely reduces the pathogenicity of *Mycobacterium tuberculosis, Coxiella burnettii, Legionella pneumophila,* and *S. typhimurium* (Braun, 2001). In fact, attempts to withhold iron by sequestering free iron during infection is a major defense strategy used by many species (Bullen et al., 2006). On the other hand, host-cells require iron for first-line defense mechanisms. Iron is needed for Haber-Weiss redox chemistry to produce hydrogen peroxide or hydroxy radicals (Eaton and Qian, 2002). Iron and proteins involved in iron metabolism have interesting links to the immune system (Hentze et al., 2004).

How the bacterium tricks the host cell into providing iron and how the host, at the same time, keeps a delicate balance between having enough iron for redox reactions and withholding iron from intracellular bacteria, however, is not well understood. Here we investigated the expression profile of macrophage iron homeostasis following the infection of RAW264.7 murine macrophages with *S. typhimurium*.

MATERIALS AND METHODS

Bacterial strains and cell

Wild-type *S. typhimurium* strain ATCC 14028 (ST) and *S. typhimurium* mutant strains *spiA::kan* (EG5793) (STA) were used. RAW264.7 murine macrophage-like cell line was obtained from ATCC.

Primers (synthesized by Bioasia Company)

The primers in Table 1 were used in this study.

Infection assay

Several colonies of *Salmonella* were collected, washed twice with cell culture complete medium (DMEM was supplemented with 10% fetal bovine serum), and resuspended with the same medium. Multiplicity of infection was adjusted to 50 using a standardized calibration curve of OD600 / colony-forming units (cfu). Bacteria were added to RAW264.7 cells in 100×15 mm cell culture dishes. Infections were initiated by centrifuging the bacteria onto the cell monolayers at 170×g for 5 min and then incubated for 1h at 37℃ 5% CO_2. In the second hour, gentamicin was added to a final concentration o f 100 μg/mL, after which, it was lowered to 10

Table 1. Primers used for real-time quantitative PCR assay.

Gene name	Accession number	Forward primer (5' → 3')	Reverse Primer (5' → 3')
Irp1	NM_007386	ACTTTGAAAGCTGCCTTGG	CTCCACTTCCAGGAGACAGG
Irp2	NM_022655	TGAAGAAACGGACCTGCTCT	GCTCACATCCAACCACCTCT
Tfr1	BC054522	TGCAGAAAAGGTTGCAAATG	TGAGCATGTCCAAAGAGTGC
Dmt1	NM_008732	GCCAGCCAGTAAGTTCAAGG	GCTGTCCAGGAAGACCTGAG
LcnR	BC062878	GCAAGGCTACCCCATACAAA	TCTTTGGGCATAGGATGGAG
Lcn2	NM_008491	CTGAATGGGTGGTGAGTGTG	TATTCAGCAGAAAGGGGACG
Steap3	BC037435	CTCTCTGTGCAGTCTCGCTG	TGCAGAGATGACGTTGAAGG
Hmox1	NM_010442	CCTCACTGGCAGGAAATCAT	CCAGAGTGTTCATTCGAGCA
Fpn1	AF226613	TGCCTTAGTTGTCCTTTGGG	GTGGAGAGAGAGTGGCCAAG
Hamp1	NM_032541	GAGAGACACCAACTTCCCCA	TCAGGATGTGGCTCTAGGCT
Ftl1	NM_010240	AAGATGGGCAACCATCTGAC	GCCTCCTAGTCGTGCTTGAG
Ftl2	NM_008049	TGACTTCCTGGAAAGCCACT	GCCTCCTAGTCGTGCTTGAG
Fth1	NM_010239	CTCATGAGGAGAGGGAGCAT	GTGCACACTCCATTGCATTC
GAPDH	NM_008084	CCCACTAACATCAAATGGGG	CCTTCCACAATGCCAAAGTT

μg/mL. At different time intervals, the RAW264.7 cells were washed three times with DMEM, and harvested for RNA analysis. Uninfected cells were used as control.

RNA analysis

Total RNA was isolated 1 h later and DNased using the Microto-Midi Total RNA Purification System (Invitrogen) according to the product instructions. RNA concentrations were determined by a RiboGreen assay (Molecular Probes). The reverse transcription reactions were carried out with 20 units of MMuLV reverse transcriptase (Fisher Scientific); 20 units RNase inhibitor (Fisher Scientific); RT-PCR buffer containing 10 mM Tris-HCl and 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM dithiothreitol, and 1 mM of each dNTP. The concentration of each reverse primer was 5 μM. 100 ng of total RNA from each sample was reverse transcribed using reverse primers. The reverse transcription reactions were incubated for 1 min at 48°C, 5 min at 37°C, 60 min at 42°C, and then 5 min at 95°C.

Real-time RT-PCR was based on the high affinity, double-stranded DNA binding dye SYBR Green using a Bio-Rad IQ SYBR Green Supermix according to manufacturer's instructions. A total of 2 μL of cDNA was used in the qPCR reactions (1×SYBR green PCR master mix, 500 nM gene specific forward and reverse primers). All qPCR reactions started with 2 min at 95°C followed by 40 cycles of 15 s at 94°C and 20 s at 55°C and 30 s at 72°C in an Applied Biosystems 7900HT Fast Real-Time PCR System, utilizing SDS 2.1 software. All reactions were run three times. Differences in mRNA concentrations were quantified by the cycles to fluorescence midpoint cycle threshold calculation ($2^{-[\Delta Ct \text{ experimental gene}-\Delta Ct \text{ housekeeping gene}]}$), using GAPDH as the housekeeping gene.

Statistical analysis

Data were expressed as mean ± SD. An analysis of variance was performed with Statview 4.0 statistical analysis software, and $P < 0.05$ was considered statistically significant.

RESULTS

The realtime PCR results are listed in Table 2.

DISCUSSION

We sought to characterize the metabolic changes of RAW 264.7 macrophage iron homeostasis following the infection with *S. typhimurium.* The SPI-2 encoded SpiA protein is a component of the outer membrane ring of the type three secretion systems (TTSS) needle complex (Tobar et al., 2006). A *spiA*$^-$ mutant strain rendered bacteria unable to translocate effector molecules into macrophage cytoplasm (Ochman et al., 1996) and displayed levels of phagosome-lysosome fusion that were much higher than those displayed by wild-type *Salmonella* and similar to those exhibited by heat-killed *Salmonella*. So we used STA as non-virulent *S. typhimurium* strain to compare with wild-type strain in studying iron metabolism. Iron is delivered to macrophages primarily through the transferrin receptor 1 (Tfr1). Tfr1 mRNA levels were significantly increased following 1 h both of ST and STA infection by 2.20-fold (F=2036.27, P=0.0001) and 1.72-fold (F=8.64, P=0.0424) as compared with non-infected RAW264.7 cells. However, after 24 h of infection, the mRNA level for Tfr1 in ST group decreased 0.7-fold (F=11.94, P=0.0259) as compared with infected 1 h, but increased 1.88-fold (F=14.11, P=0.0198) during infection with STA.

We also investigated the expression of other genes that regulate host cell iron levels by Real time PCR. There are two main eukaryotic iron-regulatory proteins, Irp1 and Irp2, which sense changes in the labile iron pool (Galy et al., 2008). They both act post transcriptionally by stabilizing their respective target mRNA and by affecting initiation of translation. When investigating Irp1 mRNA levels in RAW264.7 phagocytes, we found significant difference between controls and 1 h of two *Salmonella* strains infected cells (2.37-fold for ST, F=27.44, P=0.0063; 2.51-fold for STA, F=45.85, P=0.0025); when we compared 1 h with 24 h infected samples, there was

Table 2. mRNA expression of iron metabolism-related genes in RAW264.7 cells after infection with viable *Salmonella*.

Gene name	Uninfected	ST infection sample		STA infection sample	
		Infection 1 h	Infection 24 h	Infection 1 h	Infection 24 h
Irp1	1	2.37±0.45	2.15±0.68	2.51±0.39	0.68±0.06
Irp2	1	2.77±0.32	3.73±1.01	2.52±0.78	2.76±0.32
Tfr1	1	2.20±0.04	1.83±0.10	1.72±0.43	4.15±0.35
Dmt1	1	12.36±2.24	3.22±0.17	5.36±0.35	12.26±3.41
LcnR	1	3.06±0.41	0.66±0.12	2.23±0.36	0.24±0.05
Lcn2	1	2.07±0.38	17.44±1.41	1.18±0.15	11.63±0.54
Steap3	1	2.86±0.18	1.87±0.43	5.20±0.68	3.78±1.19
Hmox1	1	1.33±0.12	6.81±0.39	2.87±0.31	6.34±0.58
Fpn1	1	1.04±0.12	0.62±0.09	1.42±0.13	0.57±0.12
Hamp1	1	3.88±0.45	11.85±1.73	2.64±0.33	1.42±0.25
Ftl1	1	0.81±0.05	1.17±0.09	1.16±0.04	1.46±0.07
Ftl2	1	0.88±0.03	1.23±0.07	1.18±0.01	1.49±0.04
Fth1	1	0.77±0.07	1.58±0.17	1.34±0.10	1.66±0.13

Mean values shown were normalized to GAPDH mRNA levels and compared to correlated values found in uninfected macrophages (=1).

no significant difference in ST-infected cells (1.11-fold, F=0.60, P=0.4831), but Irp1 expression decreased (0.27-fold, F=3996.75, P=0.0001) in 24 h STA -infected cells. Irp2 mRNA levels was also induced (2.77-fold, F=92.96, P=0.0006) in 1 h ST-infected cells as compared with non-infected RAW264.7 cells, and no substantially alter in 24 h ST-infected cells (1.11-fold, F=0.60, P=0.4831) as compared with 1 h infection. In STA-infected samples, Irp2 mRNA levels at 1 h increased 2.52-fold (F=11.37, P=0.0280) as compared with controls, while the mRNA levels also no substantially alter (0.90-fold, F=0.40, P=0.5599) after 24 h of infection as compared with 1 h infection.

After uptake of iron and vesicle acidification, iron is reduced to its ferrous form by the ferrireductase six-transmembrane epithelial antigen of the prostate 3 (Steap3) prior to being transferred across the endosomal membrane (Ohgami et al., 2005). After 1 h of infection with wild-type *Salmonella,* mRNA levels for Steap3 were increased 2.86-fold (F=313.07, P=0.0001) as compared with controls. After 24 h infection with ST, Steap3 moderately decreased 0.66-fold (F=10.41, P=0.0321) as compared with 1 h infection. In STA infection sample, mRNA levels for Steap3 at 1 h were increased 5.20-fold (F=115.98, P=0.0004) as compared with controls, but declined at 24 h infection period (0.67-fold, F=10.85, P=0.0301) as compared with 1 h infection.

After reduction, ferrous iron is transported into the cytosol by Divalent metal transporter 1(Dmt1) (Andrews and Schmidt, 2007). After 1 h of infection with wild-type *Salmonella,* mRNA levels for Dmt1 is increased 12.36-fold (F=64.49, P=0.0013) as compared with controls. After 24 h infection with ST, Dmt1 decreased 0.27-fold (F=793.44, P=0.0001) as compared with 1 h infection. In STA infection sample, mRNA levels for Dmt1 at 1 h (5.36-fold, F=465.08, P=0.0001) were significantly different from control. This increase continued over a 24 h infection period (2.11-fold, F=14.66, P=0.0186) as compared with 1 h infection.

The lipocalin system provides the host with another way of scavenging iron. At the same time, lipocalin2 (Lcn2) can interact with bacterial siderophores to inhibit bacterial growth and has now been recognized as an important arm of the innate immune response (Flo et al., 2004). The secreted gene product Lcn2 is induced 2.07-fold (F=22.88, P=0.0088) when cells are infected with ST at 1 h as compared with controls. This increase continued over a 24 h infection period (8.6-fold, F=70.14, P=0.0011) as compared with 1 h infection. In STA infection sample, mRNA levels for Lcn2 at 1 h did not alter (1.18-fold, F=4.00, P=0.1161) as compared with control, but increased (11.63-fold, F=1183.82, P=0.0001) at 24 h infection as compared with 1 h infection. Wild type *Salmonella* also raised LcnR mRNA levels 3.06-fold (F=78.44, P=0.0009) at 1 h as compared with control, but decreased (0.21-fold, F=7956.57, P=0.0001) at 24 h as compared with 1 h infection. In STA infection sample, mRNA levels for LcnR at 1 h (2.23-fold, F=19.26, P=0.0118) were increased as compared with control, but also decreased (0.07-fold, F=76729.00, P=0.0001) at 24 h infection period as compared with 1 h infection.

Infected host cells can restrict the intracellular iron pool available for intracellular parasites by transporting iron out of the cells via ferroportin 1 (Fpn1), a transmembrane iron efflux protein (Knutson and Wessling-Resnick, 2003). Fpn1 is negatively regulated by hepcidin antimicrobial peptide (Hamp1), thus effectively reducing iron efflux (Nguyen et al., 2006). Hepcidin is significantly upregulated 3.88-fold (F=118.54, P=0.0004) in infections with ST at 1 h with accompanying unchanged in Fpn1

(1.04-fold, *F*=0.32, *P*=0.6030) as compared with control, and Hamp1 increase continued (3.1-fold, *F*=32.90, *P*=0.0046) at 24 h infection with decreased in Fpn1 (0.60-fold, *F*=192.64, *P*=0.0002) as compared with 1 h infection. Hamp1 is increased (2.64-fold, *F*=73.42, *P*=0.0010) in infections with STA at 1 h with accompanying increased in Fpn1 (1.42-fold, *F*=30.77, *P*=0.0052) as compared with control, and Hamp1 moderately decreased (0.51-fold, *F*=41.16, *P*=0.0030) at 24 h infection with decreased in Fpn1 (0.33-fold, *F*=498.78, *P*=0.0001) as compared with 1h infection.

This suggests a stronger effect for iron retention in the host cell during infection with ST. Heme oxygenase (Hmox1) catalyzes the conversion of heme to biliverdin, iron, and carbon monoxide. In macrophages it has an important antioxidative protective function, presumably by reducing pro-oxidant or proapoptotic hemoproteins (Orozco et al., 2007). The mRNA level for Hmox1 is moderately increased 1.3-fold (*F*=23.42, *P*=0.0084) in macrophages infected by ST at 1 h. This increase continued over a 24 h infection period (5.13-fold, *F*=886.43, *P*=0.0001) as compared with 1 h ST infection. In STA infection sample, mRNA levels for Hmox1 at 1h were increased (2.87-fold, *F*=109.54, *P*=0.0005) as compared with control, and the increase continued (2.21-fold, *F*=1324.96, *P*=0.0001) at 24 h infection as compared with 1 h infection.

The components of the ferritin iron storage system, ferritin heavy chain 1(Fth1), ferritin light chain 1 (Ftl1) and ferritin light chain 2 (Ftl2), are moderately decrease by infection with ST at 1 h (0.77-fold, *F*=32.39, *P*=0.0047; 0.81-fold, *F*=51.57, *P*=0.0020; 0.88-fold, *F*=61.71, *P*=0.0014, respectively) as compared with control, and increased over a 24 h infection period (2.05-fold, *F*=2037.88, *P*=0.0001; 1.45-fold, *F*=867.86, *P*=0.0001; 1.40-fold, *F*=369.23, *P*=0.0001, respectively) as compared with 1h ST infection. In STA infection sample, these three mRNA levels at 1 h were increased (1.34-fold, *F*=31.65, *P*=0.0049; 1.16-fold, *F*=45.63, *P*=0.0025; 1.18-fold, *F*=972.00, *P*=0.0001) as compared with control, and still increased (1.24-fold, *F*=576.00, *P*=0.0001; 1.24-fold, *F*=782.29, *P*=0.0001; 1.27-fold, *F*=182.25, *P*=0.0002) at 24 h infection as compared with 1 h infection.

Thus, wild-type *S. typhimurium* appears to drive an active transferrin-mediated iron uptake program after infection 1 or 24 h. This was supported by increased mRNA levels for Irp1 and Irp2, induction of genes required for transfer of iron to the cytosol via Dmt1 and Steap3, and by not too much iron efflux changing through Fpn1 and Hamp1. The *spiA*$^-$ *Salmonella* mutant strain used in our studies caused an increase in Tfr1 at 1 or 24 h, and led to decrease in Fpn1 and Hamp1 at 24 h as compared with 1 h. The assessment was that the labile iron pool increased after infection with *spiA*$^-$ *Salmonella* for 24 h. Of interest in this context is that the *spiA*$^-$ *Salmonella* mutant strain used in our studies behaves quite different to the *spiC*$^-$ strain by lacking an increase in Tfr1, but leading to an increase in Fpn1 (Pan et al., 2010). Macrophage iron overload inhibits the transcription of iNOS and the generation of NO (Weiss et al., 1994) and became one of the reasons for *Salmonella* survival inside the macrophage.

ACKNOWLEDGMENT

This work was supported by the National Natural Science Foundation of China (Contract No. 30972633).

REFERENCES

Andrews NC, Schmidt PJ (2007). Iron homeostasis. Annu. Rev. Physiol., 69: 69-85.

Braun V (2001). Iron uptake mechanisms and their regulation in pathogenic bacteria. Int. J. Med. Microbiol., 291(2): 67-79.

Bullen JJ, Rogers HJ, Spalding PB, Ward CG (2006). Natural resistance, iron and infection: a challenge for clinical medicine. J. Med. Microbiol., 55(Pt 3): 251-258.

Eaton JW, Qian M (2002). Molecular bases of cellular iron toxicity. Free. Radic. Biol. Med., 32(9): 833-840.

Flo TH, Smith KD, Sato S, Rodriguez DJ, Holmes MA, Strong RK, Akira S, Aderem A (2004). Lipocalin 2 mediates an innate immune response to bacterial infection by sequestrating iron. Nature, 432(7019): 917-921.

Galy B, Ferring-Appel D, Kaden S, Gröne HJ, Hentze MW (2008). Iron regulatory proteins are essential for intestinal function and control key iron absorption molecules in the duodenum. Cell. Metab., 7(1): 79-85.

Hentze MW, Muckenthaler MU, Andrews NC (2004). Balancing acts: molecular control of mammalian iron metabolism. Cell, 117(3): 285-297.

Knutson M, Wessling-Resnick M (2003). Iron metabolism in the reticuloendothelial system. Crit. Rev. Biochem. Mol. Biol., 38(1): 61-88.

Nguyen NB, Callaghan KD, Ghio AJ, Haile DJ, Yang F (2006). Hepcidin expression and iron transport in alveolar macrophages. Am. J. Physiol. Lung. Cell. Mol. Physiol., 291(3): L417- 425.

Ochman H, Soncini FC, Solomon F, Groisman EA (1996). Identification of a pathogenicity island required for Salmonella survival in host cells. Proc. Natl. Acad. Sci. USA., 93(15): 7800–7804.

Ohgami RS, Campagna DR, Greer EL, Antiochos B, McDonald A, Chen J, Sharp JJ, Fujiwara Y, Barker JE, Fleming MD (2005). Identification of a ferrireductase required for efficient transferrin-dependent iron uptake in erythroid cells. Nat. Genet., 37(11): 1264-1269.

Orozco LD, Kapturczak MH, Barajas B, Wang X, Weinstein MM, Wong J, Deshane J, Bolisetty S, Shaposhnik Z, Shih DM, Agarwal A, Lusis AJ, Araujo JA (2007). Heme oxygenase-1 expression in macrophages plays a beneficial role in atherosclerosis. Circ. Res., 100(12): 1703-1711.

Pan X, Tamilselvam B, Hansen EJ, Daefler S (2010). Modulation of iron homeostasis in macrophages by bacterial intracellular pathogens. BMC. Microbiol., 10: 64.

Tobar JA, Carreño LJ, Bueno SM, González PA, Mora JE, Quezada SA, Kalergis AM (2006). Virulent Salmonella enterica serovar typhimurium evades adaptive immunity by preventing dendritic cells from activating T cells. Infect. Immun., 74(11): 6438-6448.

Weiss G, Werner-Felmayer G, Werner ER, Grünewald K, Wachter H, Hentze MW (1994). Iron regulates nitric oxide synthase activity by controlling nuclear transcription. J. Exp. Med., 180(3): 969-976.