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The Role of Mitochondrial Superoxide Dismutase (SOD2) During a Coxiella Burnetii Infection

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**The Role of Mitochondrial Superoxide Dismutase (SOD2) During a *Coxiella burnetii*
Infection**

Rachel Diantha Champaigne

An undergraduate honors thesis submitted in partial fulfillment of the requirements for the

degree of

Bachelor of Science

in

University Honors

and

Biology

Thesis Advisor

Rahul Raghavan

Portland State University

2015

RESEARCH QUESTION

What is the role of Mitochondrial Superoxide Dismutase (SOD2) during a *Coxiella burnetii* infection in human cells?

ABSTRACT

Coxiella burnetii is the etiological agent of acute Q fever and highly fatal chronic endocarditis in humans. It is an intracellular bacterial pathogen that initially invades macrophages and thrives within a lysosome-like parasitophorous vacuole by modifying host processes. Expression analyses of THP-1 cells (human monocyte/macrophage cell line) using RNA-seq show differential expression of several host genes in *C. burnetii* infected cells when compared to uninfected cells. Mitochondrial Superoxide Dismutase (SOD2) expression is highly upregulated (8x) during *C. burnetii* infection as opposed to uninfected cells, but it is unknown if this expression pattern is a host immune response to suppress *Coxiella* growth or whether *Coxiella* induces it to promote its own intracellular growth. To understand the function of SOD2 during *Coxiella* infection in human macrophages, a targeted RNA silencing approach (siRNA) was used to knock down SOD2 gene expression in HeLa and THP-1 cells. An increase in the levels of *C. burnetii* infection would have indicated that the increased expression of SOD2 is a host response to control intracellular bacteria. Conversely, a decrease in *C. burnetii* numbers would have suggested that SOD2 expression is beneficial to *Coxiella* and is induced by the bacterium to promote intracellular growth. A significant transfection was not completed in the HeLa cell line, due to cytotoxicity of liposome delivery system. The change in bacterial growth in THP-1 cells that were transfected with a siRNA targeting the SOD2 transcript was insignificant. A gene expression analysis indicated that the success of the transfection was at about 30%, and therefore did not significantly affect the bacterial growth rate. When a more successful transfection method is optimized, the question can be revisited.

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CHAPTER I

INTRODUCTION

Coxiella burnetii is an intracellular pathogen that causes Q-fever and chronic endocarditis in humans.¹ *Coxiella* is able to withstand many environmental challenges, including UV light, rapid temperature change, and osmotic stresses². It does so through a stasis-type variant (small cell variant), which can be aerosolized and inhaled. It is typically transmitted to humans in agricultural areas with large populations of sheep or goats, though recent research has found evidence of transmission from wildlife to humans³. When *Coxiella* is inhaled, it is endocytosed into a phagosome (Figure 1⁴). As the phagosome fuses with an endosome and transits through the endocytic cascade, its internal pH drops due to the accumulation of hydrogen pumps as the membrane fuses and grows. At the end of the cascade, the phagosome fuses with the lysosome^{5,6}.

The lysosome is traditionally the location in which all pathogens, misfolded proteins, and other cellular debris go to be broken down and recycled for the cell's needs. It is in this organelle that *C. burnetii* thrives, and replicates into both large cell variants (LCVs), the replicative form, and small cell variants (SCV), the infective form^{7,8}. The lysosome at this stage becomes a replicative parasitophorous vacuole (PV). During the replicative phase, the host continues to send cellular waste to the lysosome (now a PV) to be degraded. *Coxiella* uses the cellular waste for its own metabolic needs⁹. As the quantity of bacteria in the PV increases, the size of the PV membrane grows by fusing with other endocytic vesicles⁹. *C. burnetii* has a type IV secretion system with which it delivers protein effectors to the host cytosol^{10,11,12}. These effectors control the host's expression of specific genes; modifying cytoplasmic filament placement, apoptotic pathway, mitotic signals, lipid production, and host immune responses¹³.

Changes in expression patterns of host genes can be determined through High Throughput RNA sequencing (RNA-seq)¹⁴, which is a process in which the whole transcriptome, or all cellular RNA present at one time, can be collected and analyzed. Previously, we performed an RNA-seq analysis of THP-1 cells infected with *C. burnetii* and identified several genes that were differentially expressed in infected cells compared to non-infected cells. Expression changes can be an immune response of the host to the pathogen¹⁵ or they can be a result effectors secreted by the pathogen via a type IV secretion system.

A small interfering RNA (siRNA) is a short nucleotide sequence that can bind to transcribed mRNA, either prior to or after the splicing out of introns. The siRNA can also bind to the active site of a targeted protein, inhibiting the active site and blocking interactions between it and other peptides; this may also modify the structure of the protein, interfering with its function.

To determine which host factors were involved in expression changes during a *Coxiella burnetii* infection in HeLa cells, McDonough et al.¹⁶ performed a whole human genome-wide siRNA knockdown, in infected and uninfected cells. This included silencing of 21,121 individual human genes in 384-well plates, then using fluorescence imaging and analysis of size and quantity of the *C. burnetii* parasitophorous vacuoles in the cells. The quantity and size of the PVs allowed for the identification of host proteins were the most important for *C. burnetii* growth within HeLa cells¹⁶.

In our RNA-seq analysis in THP-1 cells, one gene that was found to be significantly upregulated during *C. burnetii* infection of THP-1 cells is mitochondrial superoxide dismutase (SOD2)^{14,17}. We found the expression of SOD2 to be upregulated by eight times the amount of an uninfected cell. This gene was not specifically discussed in McDonough's paper, though it showed a similar amount of upregulation in their supplementary data sheets.

The SOD2 protein itself is a heterotetramer that binds one manganese ion per segment. When superoxide ions are produced through normal oxidative phosphorylation processes, SOD2 uses the manganese ion to convert the superoxide radicals (O_2^-) to hydrogen peroxide (H_2O_2) and diatomic oxygen (O_2), effectively neutralizing the reactive oxygen species (ROS). Hydrogen peroxide is then further broken down by an enzyme called catalase (figure 2).

During an infection, it is believed that the host cell makes SOD2 and stores it in the mitochondria while it initiates a full-scale superoxide attack on the pathogen, then releases its SOD2 to neutralize the superoxide after the assault so that it does not harm the host. *C. burnetii* is known to combat oxidative stress from host assault with antioxidants like glutathione, vitamins A, C, and E, and carotenoids, as well as other molecular neutralizing agents^{19,20}. It is also suspected that an acid phosphatase is secreted through *C. burnetii* type IV secretion system to neutralize the oxygen free radicals¹⁸. Superoxide dismutase could benefit the host by neutralizing the superoxide ions after the immune attack, but could instead help the pathogen if it is upregulated prior to or during the host's superoxide attack. Narasipura, Chaturvedi, and Chaturvedi showed that pathogen-derived self-upregulation of SOD2 actually contributed to the virulence of the fungi, *Cryptococcus neoformans gattii*, during an infection in mice, providing it with the ability to neutralize the host's superoxide attack¹⁹.

To determine the role of SOD2 during a *Coxiella* infection, we knocked down SOD2 expression in THP-1 cells using small interfering RNAs (siRNA) and quantified bacterial growth within the transfected and non-transfected cells. We hypothesized that if we reduced expression of SOD2 and it then decreased bacterial growth, SOD2 most probably benefits the pathogen, whereas if it increased the number of intracellular bacteria, the role of SOD2 is as the host's control mechanism for combating the *Coxiella* infection.

CHAPTER II

MATERIALS AND METHODS

***Coxiella* culture media preparation**

All work with whole-cell *Coxiella burnetii* was performed in a biosafety hood by individuals with certified training for biosafety level 2 handling. *Coxiella* media (ACCM-2) was made as outlined in Table 1. The pH was adjusted to 4.75, filtered with 0.22µm pore PES membrane syringe filter and portioned for storage (10 mL into 15mL falcon tubes). *Coxiella* cells were scraped out of frozen cryo storage tube with an inoculating loop (or specified thawed PBSS stock volume) and 1 mL ACCM-2 media in a falcon tube was inoculated. It was then put into incubator at 37°C with 5% CO₂ and 2.5% O₂ on a shaking platform, and grown for at least 3 days prior to use.

HeLa cell culture media preparation

All work with whole cell human cells took place in a cell-culture hood. For 60mm culture petri dishes, HeLa cell media was made with 4.5 mL DMEM (Dulbecco minimal Eagle's medium without L-glutamine) (Invitrogen) and 0.5 mL fetal bovine serum and supplemental L-glutamine. The DMEM and FBS were used alone, without L-glutamine or antibiotics for dilution media when splitting cells. A 9:1 ratio of DMEM:FBS with L-glutamine was used for media for infections. For regular growth of HeLa cells, a 9:1 ratio of DMEM:FBS with L-Glutamine and Pen/Strep (Penicillin/Streptomycin) was used. Transfections took place using media with only 1% FBS serum with L-glutamine, but without antibiotics.

THP-1 cell culture media preparation

THP-1 Media was prepared with Roswell Park Memorial Institute (RPMI) 1640 medium with 2 mM L-glutamine, 0.05mM beta mercaptoethanol, and 10% fetal bovine serum (FBS). Pen/Strep was added at 1%²². Media was filtered with a (0.2 µm) filter prior to use to filter out any microbes from the media. Antibiotics were not used during transfection or infection, and only 1% FBS was used during transfections.

HeLa cell freezer stock preparation

Media was pulled off of cells at ~60% confluency (approximately 1 million cells) and the media was discarded. Cells were washed with 2 mL phosphate buffered saline (PBS). The PBS was removed and cells are trypsinized with 2 mL trypsin and placed into incubator for 5 minutes. Trypsinization was verified in microscope, showing that no cells remained adhered to surface of plate. 1 mL of FBS ratio was added to the dish to stop trypsinization. Media was moved to a falcon tube. Cells were spun down to a pellet in centrifuge for 3 minutes at 500 rpm. Supernatant was removed and cells were resuspended in 1 mL of freezing media (1 mL DMSO: 2 mL FBS with L-glutamine and pen/strep: 7mL DMEM). Freezing media with suspended cells was transferred to a cryotube and placed in styrofoam in a -80°C freezer overnight and transferred to liquid nitrogen cryo freezer the next day.

THP-1 cell freezer stock preparation

Undifferentiated cells are grown to ~60% confluency (approximately 1 million cells). Media used for freezer stocks have 5% DMSO along with growth regular media. 1 mL solution was added to cryo tubes, which were put into styrofoam in a -80°C freezer overnight, and transferred to liquid nitrogen cryo freezer after 24 hours.

Thawing HeLa cells from freezer

The incubator was turned on and set to 37°C with 5% CO₂. Frozen stock was thawed at room temperature while DMEM and FBS with L-glutamine and Pen/Strep were warmed in incubator. DMEM and FBS were prepared in a 9:1 ratio. 1 mL cryo-frozen HeLa cells were obtained from the Singer Lab (Portland State University). Thawed cell stock was put into a falcon tube with only DMEM and was centrifuged at 500 RPM for 3 minutes to pellet cells. Liquid was decanted into waste and 9mL DMEM and 1 mL FBS, L-glutamine, and Pen/Strep solution are added to resuspend the cells. Solution was added to two 60 mm culture dish (5 mL per dish), which were then put into incubator set at 37 °C and 5% CO₂.

Thawing THP-1 cells from freezer

The optimum cell density was 1×10^5 cells/mL to 1×10^6 cells/mL (max 2×10^6 cells/mL). No more than 5 subsequent passages from freezer stock were performed²¹. Contents were thawed quickly and cell suspension was put into a 15 mL falcon tube with 5mL media. The tube was centrifuged at 500 rpm for 3 minutes. Supernatant was discarded, and pellet was resuspended in 10 mL media and transferred to two culture dishes (60mm culture dish=5mL). Culture was kept in incubator set at 37°C and 5% CO₂.

HeLa and THP-1 cell culture passaging

1mL cells were diluted in 4 mL total growth medium (5 mL total) and added to a 60 mm culture dish plating density of 15-75% confluency. Percentage confluency was determined by desired growth period for experiment. 100 μ L of cells are plated for each 10% desired confluency for a 60mm culture plate and 20 μ L for each 10% desired confluency for each well of a 6-well plate. The Cell density was verified in an inverted microscope, or counted with a hemocytometer on a regular optical microscope. Culture plate with cells were placed into incubator set at 37 $^{\circ}$ C, 5% CO₂, with a tray of fresh deionized water in the bottom of the incubator. Media and deionized water were changed every 2-3 days. Hela cells cannot be used for more than fifty subsequent passages from frozen stock, and THP-1 cannot be used for more than five passages, or one month's time.

Differentiation of THP-1 cells

Cells were split to desired confluency at least 24 hours prior to transfection, unless transfection took place during differentiation, or if transfection took place with suspended, undifferentiated cells. 1 μ g/mL phorbol ester (phorbol 12-myristate 13-acetate) PMA, was added to full serum media to initiate differentiation of THP-1 cells into macrophages. Differentiation took place in plates that were used for experiment, and not in regular culture dishes. Stock PMA was kept at 5000x (5mg/mL in DMSO). 2 μ L of PMA solution was used for every 10 mL cell media.

Transfection- siRNA delivery in adherent cells (HeLa or differentiated THP-1)

siRNA Buffer 5x was diluted to 1x by mixing with 4x sterile RNase-free water. Solution was pipetted up and down 3-5 times while avoiding introduction of bubbles. The solution was placed on an orbital mixer/shaker for 70-90 minutes at 37 °C and briefly centrifuged to collect solution to bottom of the tube/wells. siRNA aliquots were made for each desired siRNA to a 200nM solution in 300ul of 1x buffer. Aliquots were stored in a -20 °C freezer.

In a 2 mL tube, the transfection media was prepared with 900 ul cell culture media (without serum). The transfection reagent was added to this volume. Lipofectamine and Dharmafect reagents were tried at a variety of volumes, as an attempt to find optimal amount of transfection while maintaining a low level of cytotoxicity, but 1ul/well of Dharmafect was the final amount used that did not have the cytotoxicity as other products and amounts. Transfection media was incubated at room temperature for 5 minutes, before being distributed, 300ul to each siRNA (targeting, non-targeting, and buffer only without siRNA) aliquot, which were then incubated at room temperature for 20 minutes.

Media was aspirated from adherent cells in 60mm dish and disposed. 1mL of trypsin was added to the dish and incubated at 37 °C for 10 minutes. 0.5 mL fetal bovine serum (FBS) was added to neutralize the trypsin. The dish was scraped with a cell scraper (for THP-1 cells, HeLa cells were easily removed with trypsin alone), and newly suspended cells were counted, and

calculated to determine how much was needed to be added to achieve desired amount of cells in 6-well plate. Up to 360ul cells in media was added, and that amount was divided by three (since it is 1/3 FBS), and the remaining amount of FBS, up to 120ul, was added to 10.2mL serum-free media, for a total 1% FBS when siRNA solution was added. 1.7mL cells in media was added to prelabeled tubes, corresponding to well identifier. 300 uL from siRNA tubes were added to corresponding tubes, with duplicate samples for each siRNA. The 2mL total volume from each tube was added to its matching well. Cells were incubated at 37 °C with 5% CO₂ for 48 hours prior to infection.

Transfection in suspended cells

In a 2 mL tube, the transfection media was prepared with 900 ul cell culture media (without serum). The transfection reagent was added to this volume. Lipofectamine and Dharmafect reagents were tried at a variety of volumes, as an attempt to find optimal amount of transfection while maintaining a low level of cytotoxicity, but 1ul/well of Dharmafect was the final amount used that did not have the cytotoxicity as other products and amounts. Transfection media was incubated at room temperature for 5 minutes, before being distributed, 300ul to each siRNA (targeting, non-targeting, and buffer only without siRNA) aliquot, which were then incubated at room temperature for 20 minutes

The cells that were suspended in media in the 60mm petri culture dish were aspirated, put into a centrifuge tube, and spun down in centrifuge at 500 rpm for 3 minutes to pellet cells. 5 mL 1% media was added and pellet was resuspended. Cells were counted and calculated to desired amount of cells in 10.2 mL 1% FBS media. 1.7mL cells in media was added to prelabeled tubes.

300 uL from siRNA tubes were added to corresponding tubes, for duplicate samples. The 2mL total volume from each tube was added to each well. Cells are incubated at 37 °C with 5% CO₂ for 48 hours prior to the *Coxiella burnetii* infection. Alternatively, adherent cells were plated at specified concentration of cells (THP-1 were differentiated) one day prior to transfection in 10% FBS, media was aspirated off, and transfection was completed in 1% FBS media as outlined above.

Infection of transfected adherent cells with *Coxiella burnetii*

After 2-day knock-down, cells were infected with *Coxiella burnetii* and allowed to grow for 3 days. 100 ul of *Coxiella* culture in ACCM2 media, with at least 3 days growth, was added to 900uL cell growth media in a centrifuge tube. 10ul/well from this mixture was added to 13 mL cell media with 10% FBS. Media was aspirated off of wells, and infected media was added to wells. Plate was placed in plate bucket in centrifuge and balanced. *Coxiella* cells were spun down onto HeLa or THP-1 cell monolayer for 3 min at 500 rpm. The remaining 1 mL media, and the initial inoculate tube were saved for later quantification. These tubes were stored at -80 °C until quantification. Cells in plates were placed back in incubator at 37 °C with 5% CO₂ for 3 days.

Infection of transfected suspended cells with *Coxiella burnetii*

100ul of *Coxiella* in ACCM2 and 900 uL cell media were added to a 2 mL centrifuge tube. 10 uL/mL of this solution was added to 7 mL of cell media, vortexed to mix, and distributed to the 6 wells. Plates were incubated at 37 °C for 3 days. Inoculate and dilution media were stored at -80°C until quantified.

Quantification of bacterial growth

For adherent cells, media was removed from wells and discarded. 250uL trypsin was added to each well and incubated for 10 minutes. Wells were scraped with cell scrapers to remove any remaining adhered cells. Cells in media was removed and spun down, then resuspended in 1 mL PBS. For suspended cells, cells in media were pelleted and resuspended in 1 mL PBS. 1 mL 0.2 mm zirconia beads were added to bead-beating tubes. 1 mL cells in PBS were added to tube.

Tubes were put in bead beater for 2 minutes, cooled on ice for two minutes, and beat again for two minutes. 400 uL supernatant was removed and put into new centrifuge tube. 400 uL binding buffer was added to tube and mixed well. Complete 800 uL solution was added to column from Qiagen GeneJet DNA extraction kit²³. Column was centrifuged at 14000 rpm for 1 minute and flow-through discarded. 700 uL wash buffer was added to column and centrifuged at 14000 rpm for 1 minute and flow-through discarded. Empty column was centrifuged for 1 minute at 14000, and placed in new tube. 30uL elution buffer, prewarmed to 56 °C was added to column, incubated at room temperature for 10 minutes, then centrifuged at full speed for 1 minute. Flow through elution buffer was added back to column and spun again at full speed for one minute. 2uL of DNA was run through qPCR, using SYBR green protocol²³.

SOD2 expression analysis

Cells that had been resuspended in PBS were pelleted down and resuspended in 250 uL TRI, then processed as outlined in the TRI reagent protocol²⁶. After RNA extraction, cDNA was made using Thermo Fisher cDNA protocol²⁷. Quantitative Real Time Polymerase Chain Reaction (qPCR) was conducted with SOD2 and GAPDH primers to quantify amount of SOD2 transcripts in relation to number of cells, represented by the GAPDH housekeeping gene.

CHAPTER III

RESULTS

Table 2 shows the Ct values of fluorescence relating to amount of DNA increase per PCR cycle. The number indicates how many cycles were necessary to reach the indicated threshold. The A wells were run with primers specific to *Coxiella*, which show the amount of *Coxiella* DNA in each sample. The B wells were run with GAPDH - a human housekeeping gene - primers, which show how many copies of this human DNA were present in the sample. A2, A4, B3, and B5 were transfected with SOD2 siRNA. Samples A3, A5, B4, and B6 were transfected with a non-targeting siRNA as a control. The A6 and A7 wells were from the initial inoculate on the day of infection. The inoculate wells likely did not have enough DNA in the sample to produce a Ct value. Table 2 and Figure 3 show a small variance between the bacterial growth rates in the SOD2 siRNA and the non-targeting siRNA transfections, but the difference is not significant. Figure 4 shows a normal melt curve of both *Coxiella* and GAPDH primers.

Table 3 and Figure 4 show that the success of the transfection was approximately at 30%, and that the difference between the expression rates of SOD2 in the SOD2 siRNA vs. the non-targeting siRNA were 0.7 and 1, respectively.

CHAPTER IV

DISCUSSION

Table 2 shows that the inoculate wells likely did not have enough DNA in the sample to produce a Ct value. This is likely due to the insufficient amount of time to replicate in the inoculate that the other samples had. The variance between the samples with the SOD2 transfection, versus the non-targeting transfection, are consistent, but not significant. This is consistent with the expression analysis of the SOD2 knock-down. Since the transfection only had 30% success, it had little impact on the rate of bacterial growth.

Further testing will include optimization of transfection amounts and reagents, including liposomes and possible nanoparticle delivery systems. This would help to reduce cytotoxicity, while increasing rates of transfection. HeLa cell optimization of delivery was not reached, due to cytotoxicity of Lipofectamine and Dharmafect. THP-1 cells had a successful knock-down, but the amount of change in expression was not significant enough to reach a conclusion as to whether the previously noted 8x increase in SOD2 expression was a benefit to the host or the pathogen, or the importance of this expression change during the infection. When a more successful transfection method is optimized, the question can be revisited.

TABLES AND FIGURES

TABLES

Table 1: Coxiella ACCM-2 Media (100 mL)

Componentants	Amount
Citric acid	256.8 mg
Sodium citrate (2H ₂ O)	474 mg
Potassium phosphate	50 mg
Magnesium Chloride (6H ₂ O)	20 mg
Calcium chloride (2H ₂ O)	1.32 mg
Iron Sulfate	(1uM)
Sodium Chloride	728 mg
L-cysteine (1H ₂ O)	26.34 mg
Bacto Neopeptone	10 mg
Casamino acids	250 mg
Methyl-b-Cyclodextrin	100 mg
RPMI w/ glutamax	12.5 mL
Deionized H ₂ O	86.5 mL

Table 2: qPCR results for infected cells, transfected vs. non-transfected

Well	Well Name	Well Type	Threshold (dR)	Ct (dR)
A2	Cox 1f sod2	Unknown	7149.776	26.36
A3	cox 1g nt	Unknown	7149.776	26.63
A4	cox2e sod2	Unknown	7149.776	26.00
A5	cox 2f nt	Unknown	7149.776	26.56
A6	cox1 innoc	Unknown	7149.776	No Ct
A7	cox 1 innoc	Unknown	7149.776	No Ct
B3	GAPDH 1f sod2	Unknown	7149.776	24.83
B4	GAPDH 1g nt	Unknown	7149.776	26.41
B5	GAPDH 2e sod2	Unknown	7149.776	26.14
B6	GAPDH 2f nt	Unknown	7149.776	26.86

Table 3: Mitochondrial superoxide dismutase (SOD2) expression analysis

Raw ct values of all samples			
siRNA type	Sample 1	Sample 2	Average
sod2	32.81	31.79	32.3
nt	32.03	31.73	31.88
siRNA expression differential			
	ct	dct	fold diff
sod2	32.3	-0.5	0.7071067812
nt	31.8		1
Adjusted rate of expression to non-targeting siRNA rate			
	SOD2 expression		
sod2	0.7		
nt	1		

FIGURES

Figure 1: Intracellular lifecycle and modulation of host expression through protein effectors (Voth and Heinzen, 2007)⁴

Figure 2- SOD2-catalyzed dismutation of superoxide

Figure 3: qPCR amplification of *Coxiella* growth in SOD2 and non-targeting siRNAs transfections with *Coxiella* and GAPDH primers

Figure 4: Dissociation curves in qPCR for SOD2 and non-targeting siRNAs transfections with *Coxiella* and GAPDH primers

Figure 5: Graphical representation of SOD2 expression in SOD2 siRNA vs non-targeting siRNA transfections

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25. Reductively Labile PRINT Particles for the Delivery of Doxorubicin to HeLa Cells

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