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Obligate intracellular bacterium *Ehrlichia* inhibiting mitochondrial activity

Yan Liu[a,b], **Zhikai Zhang**[a], **Yongquan Jiang**[c], **Lihong Zhang**[a], **Vsevolod L. Popov**[a], **Jianzhi Zhang**[a], **David H. Walker**[a], and **Xue-jie Yu**[a]

[a]Department of Pathology, University of Texas Medical Branch, Galveston, Texas 77555-0609

[b]Anhui Province Center for Disease Control and Prevention, Hefei, Anhui Province, China, 230061

[c]Center for Biomedical Engineering, University of Texas Medical Branch, Galveston Texas, 77555-0609

Abstract

Ehrlichia are obligately intracellular bacteria that reside in a vacuole in the cytoplasm of phagocytes. We determined by confocal microscopy the interaction between *Ehrlichia* and mitochondria in DH82 cells to investigate the mechanism of *Ehrlichia* survival inside the phagocyte. The most remarkable finding of our study was that *Ehrlichia* morulae interacted with mitochondria and inhibited mitochondrial metabolism,. We showed that in *E. chaffeensis*-infected DH82 cells, mitochondria did not incorporate BrdU and transcriptional level of the mitochondrial gene NADPH2 was significantly reduced, indicating the inhibition of mitochondrial metabolism. This study demonstrates that *Ehrlichia* are able to inhibit mitochondrial activities, and it opens up a new avenue for the study of *Ehrlichia* pathogenesis.

Keywords

Ehrlichia; mitochondria; ehrlichiosis

1. Introduction

Ehrlichia chaffeensis causes a life-threatening disease, human monocytic ehrlichiosis, which is an emerging infectious disease prevalent in the southern, eastern, and south-central United States[1]. *E. chaffeensis* is an obligatory intracellular bacterium with a remarkable tropism for monocytes and macrophages. The primary function of the monocyte and macrophage is to destroy engulfed bacteria. However, not only are *Ehrlichia* organisms not destroyed by phagocytes, but they actually thrive inside macrophages [1]. The key to their success in survival inside the host cell is to prevent fusion of the phagosome and lysosome. Another factor that contributes to the successful intracellular survival of *Ehrlichia* is inhibition of host cell apoptosis[2]. *Ehrlichia* has a developmental cycle that takes at least 72 h to

Corresponding to: Xuejie Yu, M.D., Ph.D., Professor, Department of Pathology, University of Texas Medical Branch, Galveston, TX 77555-0609, 409 747-1786, xuyu@utmb.edu.

complete [3]. Therefore, *Ehrlichia* must prevent host cell apoptosis in response to *Ehrlichia* infection. However, the mechanism of *Ehrlichia* organisms preventing fusion of the phagosome and lysosome and the mechanisms of *Ehrlichia* inhibiting apoptosis are not known. Understanding the structure of the *Ehrlichia* vacuole and its interaction with host cell organelles is essential for revealing the mechanisms of *Ehrlichia* surviving inside phagocytes. In this study we analyzed the interaction of the *E. chaffeensis* vacuole with mitochondria. The most remarkable finding of our study is that *Ehrlichia* interact with mitochondria and inhibit mitochondrial activities.

2. Materials and methods

2.1. Cultivation of *Ehrlichia* and preparation of antigen slides for confocal microscopy

E. chaffeensis (Arkansas strain) was cultivated in DH82 cells, a canine histiocyte cell line. To prepare cell-free *Ehrlichia* inocula, *Ehrlichia*-infected cells in a T25 flask were harvested by centrifugation at 600 xg for 10 min. The pellet was resuspended in 5 ml of MEM without bovine serum, and the cells were broken with glass beads by vortexing. The cell debris was removed by centrifugation at 200 xg for 10 min. The supernatant containing cell-free *Ehrlichia* (0.4 ml) was inoculated into each well of Labtech II 8-well chamber slides (Nalgene Nunc, Naperville, Illinois), which contained a cell monolayer of DH82 cells. The 8-well chamber slides were incubated at 37 °C for 3 days in a 5% CO_2 atmosphere. *Ehrlichia*-infected cells were permeabilized with acetone-methanol (1:1) for 10 minutes at −20°C. The slides were kept at −20°C until use and were rehydrated for 1 h at room te mperature with PBS before use. The slides were blocked with 1% BSA for 1 h at room temperature and then incubated with mouse monoclonal antibodies to mitochondria (Abcam, Cambridge, MA) and subsequently with corresponding secondary antibodies for 1 hour at 37 °C each.

2.2. BrdU and aphidicolin treatment of DH82 cells

Aphidicolin inhibits DNA polymerase-*α*, and is a potent inhibitor of nuclear DNA synthesis, but has no effect on mitochondrial DNA synthesis because the replication of mitochondrial DNA requires DNA polymerase-γ not -α [4]. To observe mitochondrial DNA synthesis, two days after *E. chaffeensis* infection, infected DH82 cells or mock-infected DH82 cells were treated with aphidicolin (20 μM) (Sigma) for 1 hour, and then BrdU (Sigma) was added at a final concentration of 15 μM. After incubation at 37°C for 24 h, the cells were stained with MitoTracker Orange. Cellular DNA was denatured by incubation in 2 N HCl at 37°C for 1 h, and acid was neutralized with 100 mM borate buffer, pH 8.5. The slides were blocked with 1% BSA and incubated with a mouse anti-BrdU monoclonal antibody (Sigma) and FITC-labeled anti-mouse IgG. The cells were stained with DAPI before observation by confocal microscopy. To observe nuclear DNA synthesis, the cells were labeled with BrdU without aphidicolin treatment, and the nuclear DNA was stained with anti-BrdU antibody without DNA denaturation.

2.3. Confocal microscopy

The slides were examined with a Olympus DSU-IX81 Spinning Disk Confocal Microscope in the Center for Biomedical Engineering at the University of Texas Medical Branch. The images were analyzed using the Olympus Fluoview Ver. 2.0b Viewer.

2.4. JC-1 staining

DH82 cells cultivated in 8-chamber slides were stained with JC-1 following the instructions of the manufacturer (Invitrogen). Fluorescence of stained cells was observed using an Olympus IX70 Inverted Microscope.

2.5. Transmission electron microscopy (TEM)

T25 flasks containing monolayers of DH82 cells were inoculated with *E. chaffeensis* at an average number of 50 bacteria per host cell. The cells were incubated in Minimum Essential Medium (MEM) with 5% bovine calf serum at 37 °C with 5% CO_2 for 72 hours. The cells were fixed for TEM with 2.5% formaldehyde prepared from paraformaldehyde, and 0.1% glutaraldehyde in 0.05 M cacodylate buffer pH 7.3 to which 0.03% trinitrophenol and 0.03% $CaCl_2$ were added. After fixation the cells were washed with 0.1 M cacodylate buffer, scraped off the flasks and pelleted. The pellets were post-fixed in 1% OsO_4 in 0.1 M cacodylate buffer, *en bloc* stained with 2% aqueous uranyl acetate, dehydrated in ethanol and embedded in Poly/Bed 812 (Polysciences, Warrington, PA). Ultrathin sections were cut on a Reichert-Leica Ultracut S ultramicrotome, stained with lead citrate and examined in a Philips 201 or CM-100 electron microscope at 60 kV.

Quantitative real time PCR. RNA was extracted from *E. chaffeensis*-infected and mock-infected DH82 cells using the RNeasy Mini kit (Qiagen). One step of reverse quantitative PCR was performed using iScript One Step RT-PCR Kit with SYBR Green (Bio-Rad). PCR primers for the NADPH2 gene (GCCCACATAGGCTGAATAGC and ATAGGGTCGTGGTGGATGAG) were designed from the canine mitochondrial genome sequence (EU789780.1). Primers for the canine GAPDH gene (TGTCCCCACCCCCAATGTATC and CTCCGATGCCTGCTTCACTACCTT) were described previously [5].

3. Results

3.1. *E. chaffeensis* morulae recruited mitochondria

In uninfected DH82 cells mitochondria are evenly distributed in the perinuclear region (Fig 1). In *E. chaffeensis*-infected DH82 cells, *E. chaffeensis* morulae were surrounded by mitochondria. Actually, most of the mitochondria in the host cells concentrated around *E. chaffeensis* morulae rather than in the cytoplasm around the cell nucleus (Fig.1). Next we addressed the question whether mitochondria are specifically recruited by *E. chaffeensis*. We infected DH82 cells with *E. chaffeensis* and fluorescence-labeled 1 μm latex beads at the same time (Figure 1) Confocal microscopy showed that *E. chaffeensis* and mitochondria were colocalized, but phagocytosed beads did not colocalize with mitochondria (Fig.1). A 3D reconstruction by confocal microscopy further showed that host cell mitochondria concentrate around *Ehrlichia* morulae (Fig 2). TEM results also confirmed that most of mitochondria were closely associated with *E. chaffeensis* morulae and free mitochondria were scarce in the cytoplasm in the infected DH82 cells (Fig 3). Thus, mitochondria are specifically recruited by *E. chaffeensis*.

3.2. *E. chaffeensis* inhibit mitochondrial metabolism

We used BrdU incorporation to determine whether mitochondria surrounding *E. chaffeensis* morulae are metabolically active. The mitochondria incorporating BrdU actively synthesize DNA, and the ones that do not incorporate BrdU are not synthesizing DNA. We first labeled the nuclei of DH82 cells with BrdU without inhibition of DNA synthesis by aphidicolin. The nuclei of *E. chaffeensis*-infected and mock-infected DH82 were labeled intensely by BrdU, suggesting that *E. chaffeensis* did not inhibit DNA synthesis of host cell nuclei. Compared to the nuclei, the mitochondria contained little DNA, and mitochondria were invisible compared with the intense green color of nuclei (Figure 4A). Therefore, we used aphidicolin to inhibit DNA synthesis in host cell nuclei, which did not affect mitochondrial DNA synthesis. After aphidicolin treatment, nuclei were not stained by BrdU, but mitochondria in mock-infected DH82 cells were labeled brightly by anti-BrdU antibody, suggesting that mitochondria actively incorporated BrdU to synthesize mitochondrial DNA in uninfected

DH82 cells (Fig.4 B). Conversely, in *E. chaffeensis*-infected DH82 cells, very few mitochondria were labeled with anti-BrdU antibody, suggesting that most mitochondria in infected DH82 cells were not synthesizing DNA (Fig. 4C).

3.3. Analysis of mitochondrial metabolism by real time PCR

To determine the inhibition by *E. chaffeensis* of nuclear gene transcription and mitochondrial gene transcription, we determined the transcription levels of the housekeeping gene GAPDH (a nuclear gene) and the mitochondrial genes *nadph2* and *cytB*. Real time PCR demonstrated that the transcriptional levels of the nuclear GAPDH gene and mitochondrial genes *nadph2* and cytochrome b (*cyt b*) were decreased from the second day to the third day after infection. However, the transcriptional level of mitochondrial genes was significant ($p<0.01$) at all time points from the second day after infection compared to the nuclear GAPDH gene. (Fig. 5).

3.4. Mitochondrial membrane permeability in *E. chaffeensis*-infected DH82 cells

We further determined whether *E. chaffeensis* affected the mitochondrial membrane permeability. Mitochondrial membrane potential, $\Delta\psi m$, is an important parameter of mitochondrial function used as an indicator of cell health. In healthy cells with high mitochondrial $\Delta\psi m$, JC-1 spontaneously forms complexes known as J-aggregates with intense red fluorescence. On the other hand, in apoptotic or unhealthy cells with low $\Delta\psi m$, JC-1 remains in the monomeric form, which shows only green fluorescence [6-8]. We stained *E. chaffeensis* infected DH82 cells with JC-1 at 24, 48, 72 and 96 hours after infection. Mock-infected DH82 cells were stained at the same time points as controls. All *E. chaffeensis*-infected DH82 showed intense red fluorescence at all time points from 24 to 120 hours after infection despite the fact that all cells were heavily infected (Fig. 6). The intensity of red fluorescence was even stronger in *E. chaffeensis*-infected DH82 cells than in mock-infected DH82 cells at 120 hours. After 48 hours of infection, *E. chaffeensis*-infected DH82 monocytes were differentiated into macrophages, which firmly attached to the flask by elongated pseudopodia. Most of *E. chaffeensis*-infected cells contained multiple nuclei at 120 hours after infection (Fig. 6). At all time points uninfected DH82 cells remained as monocytes and became condensed after 72 hours (Fig. 6). These results suggest that in *E. chaffeensis*-infected DH82 cells mitochondria maintained a normal $\Delta\psi m$ and *E. chaffeensis* inhibited apoptosis of host cells.

4. Discussion

Ehrlichia morulae had been found to be surrounded by mitochondria previously by electron microscopy [9]. Here we further investigated the association of *Ehrlichia chaffeensis* with mitochondria and whether *Ehrlichia* inhibited mitochondrial activity. We demonstrated that in *Ehrlichia* infected cells mitochondria redistributed around *Ehrlichia* morulae and mitochondrial metabolism was inhibited by *Ehrlichia* infection. In Ehrlichia infected cells mitochondria maintained membrane permeability. Stabilizing mitochondrial membrane permeability may be related to inhibition of apoptosis as been demonstrated in *E. ewingii* [10]. The mechanism of *Ehrlichia* inhibition of the host mitochondrial metabolism is not known currently and needs to be further investigated *in vivo*. *Ehrlichia* might secrete proteins that interact with mitochondria to stabilize the mitochondrial membrane permeability, which results in mitochondrial hibernation. A dormant mitochondrion may not trigger apoptosis in the infected cell, or it will not compete for nutrients with *Ehrlichia*. Although mitochondria are essential for producing energy, cells can survive without mitochondria by anaerobic respiration [11,12]. We hypothesize that in *E. chaffeensis*-infected cells, the cells may survive by anaerobic respiration because of inhibition of mitochondrial activities by *E. chaffeensis*.

Mitochondria have been reported to accumulate around the parasitophorous vacuole of obligate intracellular protozoa including *Toxoplasma gondii* and the intracellular bacteria *Chlamydia psittaci* and *Legionella pneumophila* [13-15]. The observation that mitochondria accumulate around the parasitophorous vacuole conveyed the impression that *C. psittaci* acquires ATP from mitochondria [16]. However, transfer of ATP from mitochondria to the parasitophorous vacuole is not required by either bacterium associated with mitochondria. Neither *Legionella pneumophila* nor *Ehrlichia* apparently needs ATP from the host cells because their genomes encode all the enzymes involved in ATP generation in the TCA cycle and these bacteria do not possess an ATP/ADP translocase for transporting ATP [17,18]. Therefore, these observations suggest that the association of bacteria with mitochondria is not for acquisition of ATP. Our study demonstrated that *Ehrlichia* recruit mitochondria to manipulate mitochondrial metabolism, which may results in inhibition of host cell apoptosis [19]. One mechanism utilized by *Chlamydia* to prevent apoptosis is the use of a secreted product to prevent cytochrome *c* release [19]. We hypothesize that the close apposition of mitochondria and intracellular bacteria such as *Ehrlichia*, *Chlamydia* and *Legionella* is required for bacteria to effectively deliver proteins to mitochondria to inhibit the mitochondrial apoptosis pathway.

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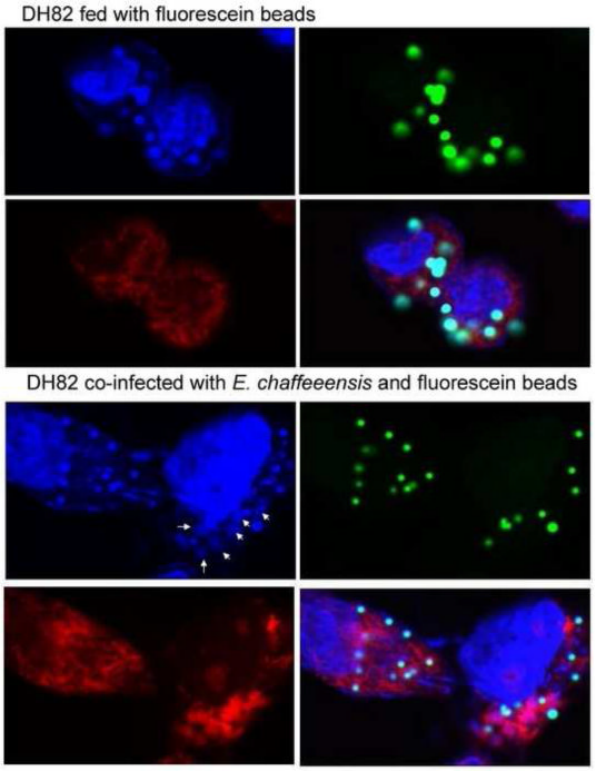

Fig. 1.

Confocal microscopy of *E. chaffeensis*-infected DH82 cells. DH82 cells were co-infected with *E. chaffeensis* and fluorescein-labeled 1 μm plastic beads. At 72 hours after infection, cells were stained with DAPI and antibody to mitochondria. The confocal images were (A) DAPI-stained nuclei and ehrlichial morulae (Blue); (B) fluorescein-labeled beads (green); (C) Antibody stained mitochondria (Red); , and (D) merged image of A, B, and C. Mitochondria in the cells were colocalized with *E. chaffeensis* morulae (arrow), but did colocalize with plastic beads.

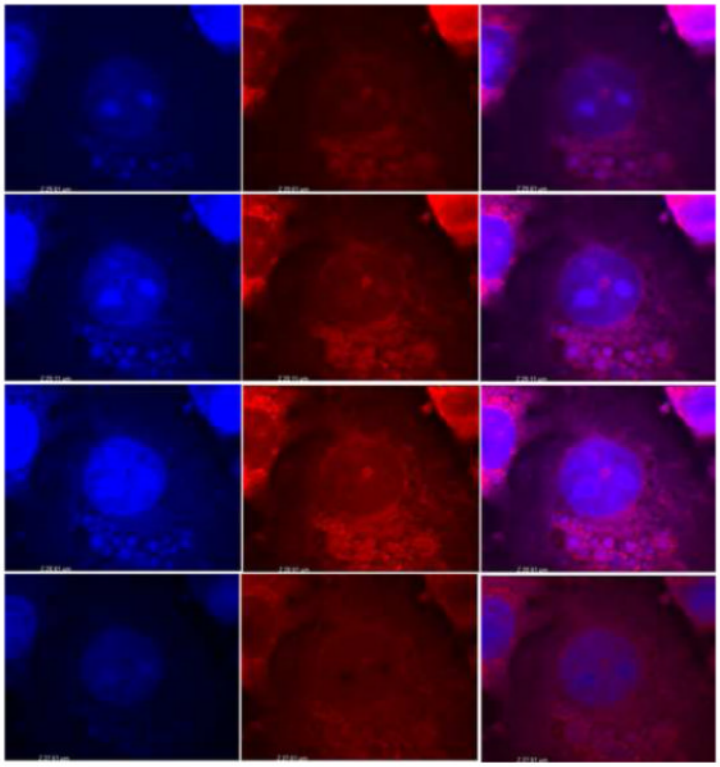

Fig. 2.
The 3D reconstruction *E. chaffeensis* infected DH82 cells. Nuclei and *Ehrlichia* were stained by DAPI (Blue) and mitochondria were stained with antibody specific to mitochondria (Red). Three sections (29.61 μM, 29.11 μM, and 28.11 μM) were shown to indicate that *E. chaffeensis* are associated with mitochondria.

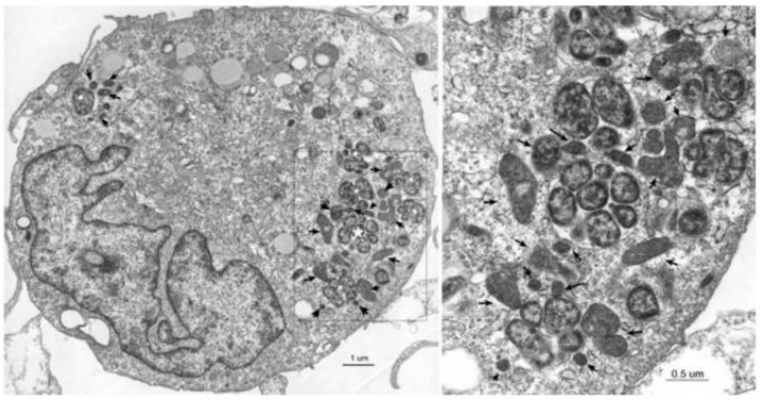

Fig 3.

Electron micrograph of *E. chaffeensis* infected DH82 cells. Arrows indicate mitochondria, and white stars indicate ehrlichial morulae. The area in the box in the picture on the left was enlarged in the picture on the right.

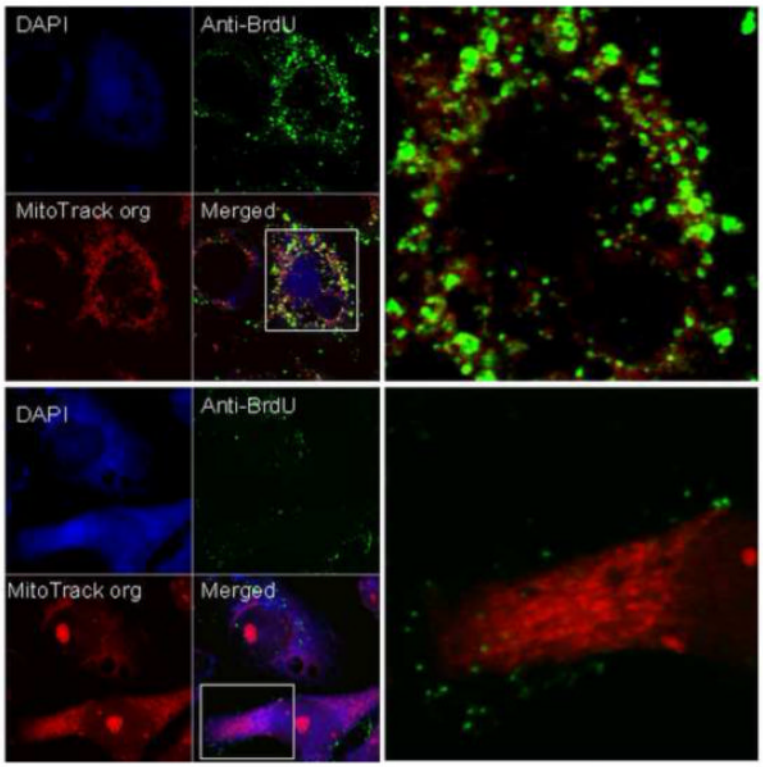

Fig.4.

Confocal microscopy of BrdU-labeled DH82 cell nuclei and mitochondria. In all pictures, blue color is DAPI-stained nuclei or *E. chaffeensis* (arrows), red is MitoTrack orange stained-mitochondria and green is BrdU-labeled nuclei or mitochondria. A, *E. chaffeensis*-infected (left) and mock-infected DH82 cells without aphidicolin treatment. B and C, *E. chaffeensis*-infected and mock-infected DH82 cells were treated with aphidicolin. The areas in the boxes are enlarged on the right.

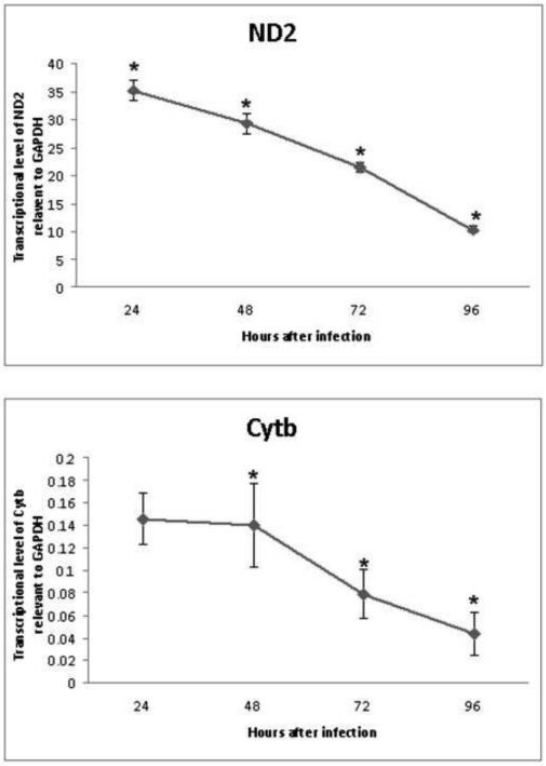

Fig 5.

Real time PCR analysis of *E. chaffeensis* inhibition of nuclear and mitochondrial gene transcription. RNA was extracted from DH82 cells harvested at 24, 48, 72, and 96 hours after *E. chaffeensis* infection and used as template for reverse transcription. The cDNA was amplified by real time PCR using primers from the nuclear GAPDH gene and mitochondrial genes *nadph2* (ND) and *cyt b*. The relevant transcriptional level of mitochondrial gene were calculated by the transcriptional level of mitochondrial gene/ the transcriptional level of nuclear gene GAPDH. * indicating significant difference of transcriptional levels between mitochondrial NADPH or Cyt b gene and nuclear GAPDH gene.

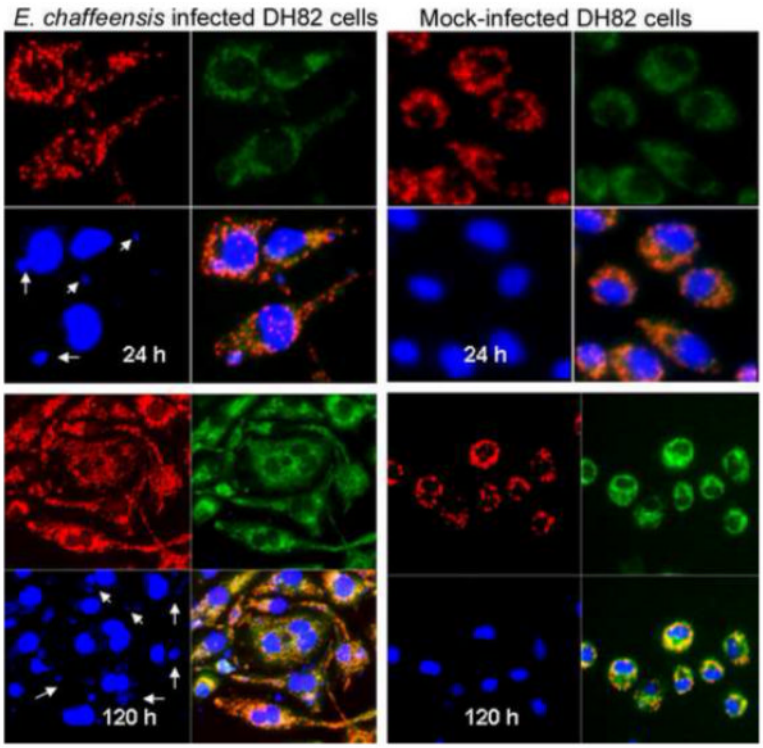

Figure 6.

JC-1 stained *E. chaffeensis* infected DH82 cells. The images are representatives of *E. chaffeensis*-infected or mock-infected DH82 at 24 and 120 hours after infection. Red fluorescence shows JC-1 stained mitochondria; green florescence shows JC-1 stained cytoplasm; and blue color is DAPI-stained nuclei and *E. chaffeensis* morulae (arrows). The image on the bottom-right of each panel is the merged picture. Mock-infected DH82 cells remain as monocytes and became condensed at day 5, but *E. chaffeensis*-infected DH82 cells became elongated macrophages with multiple nuclei in each cell, suggesting that the infected cells were still multiplying after 5 days of infection in spite of heavy infection with *E. chaffeensis*. The panels on top are at the same magnification, and the panels on bottom are at the same magnification.