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Host metabolism promotes growth of *Chlamydia pneumoniae* in a low oxygen environment

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ABSTRACT

Chlamydia pneumoniae infections of the respiratory tract are common and are associated with acute and chronic diseases such as community-acquired pneumonia (CAP) and chronic obstructive pulmonary disease (COPD). Recent studies have shown that reduced environmental oxygen availability promotes chlamydial growth in infected host cells. The underlying mechanisms remain unclear. We performed a targeted siRNA screen coupled with an automated high-throughput microscopic analysis to identify key host cell genes that play a role in promoting the hypoxic growth of *C. pneumoniae*. A total of 294 siRNAs – targeting 98 selected genes including central mediators of metabolic, trafficking and signaling pathways – were tested on chlamydial inclusion formation in *C. pneumoniae* infected A549 cells under normoxic (20% O_2) and hypoxic (2% O_2) conditions 48 h post infection. Evaluation of the different functional clusters of genes revealed that under hypoxic conditions, enhanced growth of *C. pneumoniae* was centrally mediated by the host cell glycolytic pathway. Inhibition of the phosphofructokinase (PFK), lactate dehydrogenase (LDH), glycerol-3-phosphate dehydrogenase (GPD2) and the forkheadbox O3 (FOXO3) gene-expression by siRNAs abrogated chlamydial progeny. The pivotal role of host cell glycolysis in chlamydial development under hypoxia was further confirmed by pharmacological inhibition of the pathway by 2-fluoro-deoxy-glucose. The results indicate that the microenvironment of the host cell determines the fate of *C. pneumoniae* by controlling pathogen-induced metabolic pathways.

Introduction

Chlamydia pneumoniae causes acute infections of the upper and lower respiratory tract and has been linked to the pathogenesis of chronic obstructive pulmonary disease (COPD), asthma and atherosclerosis (Hahn et al., 2002; Watson and Alp, 2008). Exposure to *C. pneumoniae* frequently occurs in children or young adults resulting in seropositivity against *C. pneumoniae* of more than half of the population by age 20 (Grayston, 1992). *C. pneumoniae*, an obligate intracellular bacterium, strictly depends on the host cell to support its growth.

Infections by intracellular pathogens activate diverse host cell signaling pathways that depend on the type of the pathogen, the type of host cell and the environmental conditions. Differential host cell gene regulation within the Chlamydiae genus was demonstrated by microarray analysis in cells that were infected by *C. pneumoniae* and *C. trachomatis* (Hess et al., 2003). The effect of environmental oxygen concentrations on the gene expression changes of *C. pneumoniae*-infected cells has not been studied so far, although oxygen concentrations profoundly affect *C. pneumoniae* development (Juul et al., 2007; Rupp et al., 2007). Oxygen concentrations vary dramatically between different organ tissues under physiological conditions ranging from 1 to 11% throughout the human body (Carreau et al., 2011). In case of a chronic inflammatory process, oxygen availability may further decrease due to increased oxygen consumption of infiltrating inflammatory cells and the high metabolic turnover of inflamed tissues (Eltzschig and Carmeliet, 2011). *C. pneumoniae* growth is significantly increased under low oxygen concentrations (O_2 < 3%) (Juul et al., 2007; Rupp et al., 2007). Moreover, hypoxia not only directly affects intracellular chlamydial development, but also impairs the anti-chlamydial effector mechanisms of the host cells. The anti-chlamydial activity of interferon-γ (IFNγ) is reduced under hypoxia. This prevents IFNγ-induced eradication or persistence of the bacteria that is normally observed under normoxic conditions (Roth et al., 2010).

Infections with human pathogens have been shown in general to stabilize the alpha subunit of the hypoxia inducible factor-1 (HIF1A) and to increase transcriptional regulation of HIF1A

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mediated genes, as a result of enhanced cellular oxygen consumption (Werth et al., 2010). HIF1A has been shown to be essential for *C. pneumoniae* growth under hypoxic conditions (Rupp et al., 2007). HIF1A controls the transcription of a plethora of genes involved in energy metabolism, differentiation, cell proliferation, apoptosis and angiogenesis (Rankin and Giaccia, 2008; Schodel et al., 2011). HIF1A was suggested to positively mediate host cell metabolic changes in *C. pneumoniae*-infected cells such as glucose uptake (Rupp et al., 2007). HIF1A is also stabilized under normoxic conditions in *Chlamydia*-infected cells (Rupp et al., 2007; Sharma et al., 2011). Hypoxic stabilization of HIF1A and normoxic stabilization by Toll like-receptor ligands can regulate distinct gene expression patterns (Jantsch et al., 2011).

There is a dearth of information about cellular factors other than HIF1A stabilization that would play a role in promoting *C. pneumoniae* infection under hypoxia. This lack of information prompted us to design a siRNA screen. We silenced genes that are involved in metabolism, trafficking and signaling in a pulmonary epithelial cell line infected with *C. pneumoniae* under normoxic (20% O_2) or hypoxic (2% O_2) conditions. The effects of silencing on chlamydial inclusion formation were measured by high-throughput microscopic analysis after immunostaining of chlamydial inclusions. The screen revealed that differentially regulated metabolism under hypoxia plays a central role in supporting *C. pneumoniae* infection.

Materials and methods

Cell culture and infection with C. pneumoniae

A549 cells (ACC 107, DSMZ, Braunschweig, Germany) were cultured in Dulbecco's modified Eagle's Medium (DMEM) with glucose (4.5 g/l) (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 4 mM L-glutamine, 110 mg/l sodium-pyruvate, 10 mg/ml gentamicin and 30 mM Hepes. Cells were grown at 37 °C, in 5% CO_2 and 20% O_2 (humidified air) for normoxic studies or in 5% CO_2 and 2% O_2 for hypoxic studies. *C. pneumoniae* CWL029 strain (ATCC VR-1310) was purified from HEp-2 cells (ATCC CCL-23). A549 cells were infected with *C. pneumoniae* at an infection rate of 15 inclusion-forming units (IFU) per cell 6 h after siRNA transfection, in the presence of 0.1 μg/ml cycloheximide by using centrifugation for 45 min at 2000 rpm at 30 °C.

siRNA design

Three unique sites within the coding sequence of each human genes were selected (Cenix Bioscience GmbH, Dresden, Germany) and 21-mer oligonucleotides synthesized (Ambion Inc/Invitrogen).

High throughput siRNA screening

A549 cells were seeded at 2600 cells per well in 30 μl of DMEM in 384 well plates. After 48 h, medium was changed and 10 μM siRNA in 2 μl OPTI-MEM and 0.4 μl Oligofectamine (Invitrogen, Grand Island, NY, USA) was added directly to the cells and cells were infected with *C. pneumoniae* as described above. Each siRNA was transfected in triplicate in three different plates. After infection, cells were incubated under normoxic (5% CO_2 20% O_2) or hypoxic (5% CO_2 2% O_2) conditions. After 48 h incubation, cells were fixed in ice-cold methanol and stained with 4,6-diamidinio-2-phenylindole (DAPI). *C. pneumoniae* inclusions were stained with anti-LPS antibody, and host cells with an anti-tubulin antibody and appropriate fluorescent (FITC and Alexa Fluor 555) secondary antibodies.

Automated imaging and quantification

Images were acquired with a Discovery1 automated fluorescence microscope (Molecular Devices Corporation, CA, USA) using a 10× lens. In each well, cell nuclei, tubulin and chlamydial LPS were imaged in 4 fields. Image data was analyzed using a custom MetaMorph (Molecular Devices Corporation, CA, USA) based algorithm extracting the following values for each imaged field: nuclear condensation as measured by the size and number of nuclei per imaged field (DAPI staining), number of cells as measured by tubulin staining and number and size of chlamydial inclusions as measured by LPS staining. Within each field, the numbers of chlamydial inclusions were normalized to the cell numbers (infection rate). Infection rates were averaged between the 4 imaged fields within each well. Mean and standard deviations were calculated for each experimental triplicate.

Selection of hits

To rule out enhanced cytotoxicity of certain siRNA which could potential result in false positive hits, we excluded data from the evaluation where total cell numbers were low. Toxicity cut off filter of less than 200 cells/microscope field (≤30% of the mean of numbers of nuclei per microscopy field) was used to exclude data of high cytotoxicity (Supplementary Fig. 2A). This resulted in the exclusion of only 4.53% of the data from the further analysis and it caused an average of only 0.31 ± 0.04% changes in the infection rates which confirms that the results are generally not artifacts of high cell toxicity (Supplementary Fig. 2B). To select the hit siRNAs, a multi-step analysis of the data was performed (Supplementary Fig. 4A). First, the whole data set was normalized to the negative control scRNAs (100%) in each experimental screen (screen 1 and screen 2) in each condition (normoxia and hypoxia) separately. As the threshold for selecting siRNAs, to define inhibition or increase of *C. pneumoniae* infection, we set the value of two standard deviations of the negative control siRNAs. siRNAs were selected from each experimental run separately (Supplementary Fig. 4B). As a second step, to ensure reproducibility of the results, the effects of the selected siRNAs were compared between the two screens. Infection rates from the first screen were plotted against the infection rates from the second screen. Scoring values were assigned by step ranking of results by one standard deviation compared to negative control siRNA values (Supplementary Fig. 4C). Hits were selected based on their added scoring points from the two screens. The cut off filters to select hits were chosen to include approximately the top *ca.* 5% of the siRNAs as hits (added scoring point −4 and below for siRNAs that caused decreased infection and added scoring point +9 and above for siRNAs that caused increased infection).

Real-time PCR

A549 cells were seeded at 5×10^4 cells per well of a 24-well culture plate and transfected with siRNA (3 nM) and infected with *C. pneumoniae* under normoxic or hypoxic conditions as described above. After 48 h of infection, total RNA was isolated from two wells by using the NucleoSpin RNA II kit (Macherey-Nagel, Duren, Germany) and reverse-transcribed into cDNA (RevertAid First Strand cDNA Synthesis kit, Fermentas, St. Leon-Rot, Germany). PCR amplification was performed by using the LightCycler Detection System (Roche Molecular Biochemicals). Relative quantification of GPD2, LDHA, PFKP and FOXO3 mRNA expression was performed against the endogenous control β-actin gene using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001). Detection of *C. pneumoniae* tuf mRNA expression was used to analyze relative chlamydial gene expression (Maurer et al., 2007). Primers were designed for the selected genes using Primer3 (http://frodo.wi.mit.edu/primer3/).