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Contact of *Chlamydophila pneumoniae* with type II cell triggers activation of calcium-mediated NF-κB pathway

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Abstract

Nuclear factor-κB (NF-κB) plays an important role in inflammation, proliferation and regulation of apoptosis. The purpose of the present study on type II cells was to investigate whether *Chlamydophila pneumoniae* contact induces (I) a Ca^{2+} release, that (II) disrupts F-actin/β-tubulin cytoskeletal association with NF-κB/IκBα, leading to (III) a subsequent NF-κB activation.

Incubation of rat type II pneumocytes with *C. pneumoniae* caused an intracellular calcium release within seconds. Confocal laser scanning microscopy (CLSM) revealed that bacterial contact with cell surface leads to a disappearance of the microvilli and disturbs the co-localization between F-actin and NF-κB (p65). Using semi-quantitative CLSM, we show that at 10–30 min IκBα was decreased and p65 or p50 was simultaneously translocated from cytoplasm to the nucleus, resulting in a 19-fold and 17-fold increase versus control cells. During this time no bacteria were internalized into type II cells. The pre-treatment of cells with BAPTA-AM inhibited *C. pneumoniae*-mediated calcium release. BAPTA-AM or SN50 prevented the *C. pneumoniae*-induced changes in F-actin cytoskeleton and inhibited NF-κB activation. Paclitaxel reduced *C. pneumoniae*-mediated changes of β-tubulin cytoskeleton and activation of NF-κB. These results suggest that calcium-mediated cytoskeleton reorganization is involved in *C. pneumoniae*-induced NF-κB activation in type II cells.

Keywords: *Chlamydophila pneumoniae*; Calcium; Cytoskeleton; NF-κB

1. Introduction

The Gram-negative bacteria *Chlamydophila pneumoniae* (*C. pneumoniae*) cause respiratory infections [1]. *C. pneumoniae* are known to enter and persist in type II pneumocytes. We have recently shown that intracellular bacteria inhibit the surfactant transport in parallel with changes in tubulin network [2]. However, the initial process of adhesion and internalization of *C. pneumoniae* with a subsequent stimulation of cellular responses in type II cells is still poorly understood.

In endothelial and muscle cells, *C. pneumoniae* infection causes an activation of nuclear factor-κB (NF-κB) [3,4]. NF-κB is a key factor for the control of different cellular pathways and is involved in inflammation, proliferation and regulation of apoptosis. NF-κB RelA (p65) and NF-κB1 (p50) subunits are sequestered in an inactive cytoplasmatic complex by binding to its inhibitory proteins of the IκB family. Upon various stimuli, inhibitory proteins of IκB are phosphorylated and rapidly degraded. In the absence of IκB, free NF-κB translocates to the nucleus and subsequently activates the transcription of various genes [5].

NF-κB is stimulated by various extracellular signals that induce a rapid mobilization of intracellular calcium stores [6–9].

Microvilli and their cytoskeletal core protein, F-actin, are involved in cellular calcium signaling processes [10]. Some bacteria, such as *C. pneumoniae*, induce the accumulation

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of microvilli. These accumulations are used by bacteria to adhere or enter host cells [11–13].

Reorganization of structural components within the cytoskeleton is involved in activation of NF-κB. IκBα interacts with cytoskeleton-associated protein through its signal response domain [5,14].

The purpose of the present study on type II cells was to investigate whether *C. pneumoniae* contact induces (I) a Ca^{2+} release, that (II) disrupts F-actin/β-tubulin cytoskeletal association with NF-κB/IκBα, leading to (III) a subsequent NF-κB activation.

2. Materials and methods

2.1. Cell culturing and reagents

C. pneumoniae strain TW183 was cultured/purified as described recently [4]. Type II cells were isolated from the lungs of adult male Wistar rats (body weight 120–140 g) according to Dobbs et al. [15]. The cells were predominantly about 94% type II cells (n=20 experiments). The viability of cells before infection was about 98% (n=20 experiments). Isolated type II cells were pre-incubated for 30 min with either phalloidin (10 μg/ml), which stabilizes F-actin; nocodazole (10 μM), which promotes tubulin depolymerization; paclitaxel (5 μM), which inhibits tubulin disassembly; calcium chelator BAPTA-AM (5 μM); NF-κB SN50 (50 μg/ml), a cell-permeable peptide which inhibits the nuclear translocation of NF-κB Rel complexes; SN50M (50 μg/ml), a cell-permeable inactive control peptide (all chemicals were obtained from Calbiochem Darmstadt, Germany), or modified Eagle's medium (MEM) (PAA Laboratories GmbH, Linz, Austria) or with corresponding concentration of dimethyl sulfoxide (DMSO, obtained from Sigma, Deisenhofen, Germany). Thereafter, cells were incubated with or without *C. pneumoniae* for different periods of time (for details, see Results). Cells were incubated with a multiplicity of infection (MOI) of 2 (2×10^6 inclusion-forming units (IFU) per 1×10^6 target cells). Viability (determined by trypan blue exclusion) of the *C. pneumoniae*-incubated cells (about 96% n=20 experiments) was not different from control cells without bacteria.

2.2. Measurement of lactate dehydrogenase (LDH)

As an index for cell toxicity, the release of LDH was measured. After pre-incubation of cells with phalloidin (10 μg/ml), nocodazole (10 μM), paclitaxel (5 μM), BAPTA-AM (5 μM), SN50 (50 μg/ml) or SN50M (50 μg/ml) for 30 min and incubation with *C. pneumoniae* for 60 min and incubation of control cells in MEM or with corresponding concentration of DMSO cellular medium was removed. The cells were disrupted using 0.2% Triton-X (Sigma). The enzyme activity in medium and in disrupted cells was determined automatically by Synchron-LX-Systems (Beckman Instruments GmbH, Unterschleissheim, Germany). Measurements of LDH revealed no significant difference between control cells and cells after pre-treatment with drugs. Extracellular LDH concentration was always less than 4%.

2.3. Measurement of calcium flux

Three-hour adherent type II cells were washed with HANKS buffer (PAA Laboratories) and transferred into bath buffer (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose, 10 mM Na-Hepes, pH 7.4) with 2.5 μM Fluo-4 AM (Molecular Probes, Eugene, OR USA) at 37 °C for 30 min in an incubator ±with BAPTA-AM (5 μM), and than washed again twice with bath buffer (without Ca^{2+}) to remove external Ca^{2+}.

Real-time fluorescent images were obtained using an Olympus IX50 microscope equipped with a fluorescein filter set (DM 505, BA 515), Plan Neon 40×/0.9 water immersion objective (Zeiss, Jena, Germany), monochromator, Sensicam camera and imaging system (TILL Photonics GmbH, München, Germany) at excitation of 488 nm. Frames were collected at 2-s intervals following the application of *C. pneumoniae* (MOI=2) or only with bath buffer without Ca^{2+}. Fluorescence signals were recorded from two to seven cells/field.

For experiments dealing with influence of electric field on calcium signal, we used a Cytocon™ 300 system equipped with a Loader chip (Evotec Technologies GmbH, Hamburg, Germany) as described by Müller et al. [16]. Cells suspended in bath buffer (w/o Ca^{2+}) loaded with Fluo-4 and treated as described above, were injected and streamed with syringe pump in one channel of a microfluidic device with two cross-like channels. A single cell was caged in the middle of the cross with Loader cage using rf electric fields about 700 kHz and 2.5 V rms. A few seconds later, a protocol taking fluorescence images for 60–120 s (each 1 s, for 40 ms exposure time) was started. During continuous registration of intensity, the flow of the first channel was stopped and the flow of the second channel rinsed the *C. pneumoniae* on the caged cell. Fluorescence signals were recorded.

We used TILL imaging software (TILL Photonics) to analyze fluorescence intensity of the cell (region of interest).

2.4. Western blot analysis

Isolated type II cells were incubated with *C. pneumoniae* in suspension. Nuclear and cytoplasmatic extracts were made according to the methods described previously by Altavilla et al. [17]. The protein content of cytoplasmatic and nuclear extracts was determined by Bradford assay using bovine serum albumin (BSA) as a standard method (Bio-Rad Laboratories protein assay kit, Richmond, USA). Protein fractions were stored at −80 °C. IκBα protein was assayed in cytoplasmatic extracts and NF-κB p65 protein in