INTRODUCTION

Chlamydophila pneumoniae (Chlamydia pneumoniae), a Gram-negative obligately intracellular bacterium, is a widespread human pathogen causing respiratory tract infections, including pneumonia. Recently, chronic C. pneumoniae infection has been proposed as a trigger and promoter of inflammatory reactions and development of vascular lesions (Krull et al., 1999). The association of C. pneumoniae with the pathogenesis of atherosclerosis and coronary artery disease has been suggested by seroepidemiological data and detection of the organism in atheromas by culture, PCR, electron microscopy, immunohistochemistry and in situ hybridization (Saikku et al., 1988; Shor et al., 1992; Kuo et al., 1993; Campbell et al., 1995; Jackson et al., 1997; Muhlestein, 1998). The ability of C. pneumoniae to readily infect vascular cells, including smooth muscle cells, endothelial cells, macrophages and monocytes, and to stimulate these cells to produce cytokines, chemokines and adhesion molecules has been demonstrated (Godzik et al., 1995; Gaydos et al., 1996; Kaukoranta-Tolvanen et al., 1996; Quinn & Gaydos, 1999). These data support an association of an infectious process in the initiation and progression of atherosclerosis or in plaque instability.

The signal transduction pathways induced by C. pneumoniae and the molecular mechanisms of bacterium–host interaction have been elucidated recently in different cells, relevant to atherogenesis. C. pneumoniae-infected endothelial cells have been demonstrated to express endothelial adhesion molecules, up-regulate phosphorylation of p44/p42 mitogen-activated protein kinase (MAPK) and activate/translocate nuclear factor-κB (NF-κB) (Krull et al., 1999). C. pneumoniae and chlamydial heat-shock protein 60 (HSP60) were shown to stimulate proliferation of human vascular smooth muscle cells and trigger p44/p42 MAPK activation (Sasu et al., 2001). Chlamydial and human HSP60 were also reported to activate endothelial cells, smooth muscle cells and macrophages, inducing expression of adhesion molecules, production of proinflammatory cytokines and activation of NF-κB (Kol et al., 1999; Bulut et al., 2002). However, there are no data about the molecular interactions of C. pneumoniae with human fibroblasts.

Chlamydophila pneumoniae induces p44/p42 mitogen-activated protein kinase activation in human fibroblasts through Toll-like receptor 4

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Chlamydophila pneumoniae, an obligately intracellular Gram-negative bacterium and a common causative agent of respiratory tract infections, has been implicated in the induction and progression of atherosclerosis and coronary artery disease. In this study, the signalling mechanism of C. pneumoniae in human fibroblasts, a prominent cell population in chronic inflammation and persistent infection, contributing to plaque formation, was investigated. C. pneumoniae elementary bodies were demonstrated to up-regulate the phosphorylation of p44/p42 mitogen-activated protein kinase (MAPK) in human fibroblasts. The effect was independent of the chlamydial lipopolysaccharide and was likely to be mediated by a heat-labile chlamydial protein. Furthermore, an anti-Toll-like receptor 4 (TLR4) antibody was shown to abolish C. pneumoniae-induced cell activation, whereas an anti-TLR2 antibody had no effect, indicating the role of TLR4 in p44/p42 MAPK activation. Ca2+/calmodulin-dependent protein kinase inhibitor KN-62 and phosphodiesterase 4 (PDE 4) inhibitor Rolipram enhanced C. pneumoniae-induced MAPK phosphorylation and attenuated C. pneumoniae infectivity in vitro. Together the results indicate that C. pneumoniae triggers rapid TLR4-mediated p44/p42 MAPK activation in human fibroblasts and chemical enhancement of MAPK phosphorylation modulates in vitro infection at the molecular level.
inflammation and persistent infection, contributing to plaque formation and evolution during atherogenesis. They are involved in the synthesis of collagen and cytokines and are engaged in complex interactions during the inflammatory process. In our previous work we demonstrated the cellular proliferation and p44/p42 MAPK activation induced by C. pneumoniae in mouse connective tissue fibroblast cell line L-929 (Haralambieva et al., 2002a). However, the signalling mechanisms may differ depending on the cell type, the species and the origin of the cells (Means et al., 2000b; Chang et al., 2002). In this study, we investigated the signalling pathways triggered by the respiratory pathogen C. pneumoniae in human lung-derived fibroblasts and furthermore elucidated the receptor mechanism involved and the effect of new inhibitors on C. pneumoniae-induced signal transduction and infectivity.

METHODS

Cell culture and reagents. Human diploid culture from embryonic lung fibroblasts (P-742) was kindly provided by the National bank of cell lines and micro-organisms, Sofia, Bulgaria. The cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; Sigma), supplemented with 10% fetal bovine serum, CELLect Gold (ICN Pharmaceuticals), 2 mM l-glutamine and antibiotics.

Selective upstream MAPK kinase 1/2 inhibitor PD98059, protein kinase C (PKC) inhibitors Go6976 and Go6983, protein kinase A (PKA) inhibitor H-89 and Ca2+/calmodulin-dependent protein kinase (CaM kinase) inhibitor KN-62 were kindly provided by Debiopharm. Rolipram, an inhibitor of phosphodiesterase 4 (PDE 4), was kindly provided by Dr Markova, Department of Pharmacology, Medical University of Sofia, Bulgaria. Mouse monoclonal antibody p-ERK1 (E-4) specific for Tyr-204 phosphorylated ERK1 and ERK2, rabbit polyclonal antibody ERK1 (K-23), reactive with both ERK1 and ERK2, and rabbit polyclonal antibodies specific for Toll-like receptor 2 (TLR2) (H-175) and TLR4 (H-80) were purchased from Santa Cruz Biotechnology.

Micro-organisms and antigens. C. pneumoniae (TWAR) strain AR-39 (ATCC no. 53592) was obtained from the American Type Culture Collection (Manassas, VA, USA). The micro-organism was propagated in HEp-2 cells and a stock of purified elementary bodies (EBs) was obtained as described previously (Haralambieva et al., 2002a). In some cases EBs were heat-treated at 100°C for 20 min. The LPS antigen from Salmonella minnesota R595 of Re chemotype (LPS-Re) was a kind gift from C. Galanos, Max-Planck-Institute for Immunobiology, Freiburg, Germany.

Immunoblotting analysis. The immunoblotting of p44/p42 MAPK was carried out as described previously (Iankov et al., 2002). Where indicated 2.5 × 10⁵ P-742 cells were treated with 50 nM PD98059, 1 μM Go6976, 1 μM Go6983, 5 μM H-89, 10 μM KN-62, 10 μM Rolipram or anti-TLR2 and anti-TLR4 antibodies for 1 h. Cells were stimulated with 10⁶ inclusion-forming units (IFU) C. pneumoniae EBs ml⁻¹ at an m.o.i. of 4 (viable or heat-inactivated) or with 10 μg LPS-Re for the indicated periods of time. For mock infections (controls), the cells were incubated with a preparation of uninfected HEp-2 cells, purified by the same protocol used for chlamydiae. After immunoblotting, detection was carried out as described previously with some modifications (Haralambieva et al., 2002b). Confluent P-742 cell monolayers, grown as cover slip cultures, were treated for 1 h where indicated with 10 μM Rolipram or 10 μM KN-62 and inoculated with 10⁵ IFU C. pneumoniae ml⁻¹ without centrifugation. The plates were incubated for 2 h at 37°C, washed five times with prewarmed RPMI and overlaid with 1 ml RPMI without cycloheximide, containing kinase inhibitors where indicated. After 48 h incubation at 37°C in a CO₂ atmosphere, the monolayers were fixed in methanol and stained for immunofluorescence with a genus-specific monoclonal antibody. The IFU were counted in 100 fields at a magnification of ×400 and the results were presented as percentage infectivity in IFU in comparison with the control IFU (100%).

RESULTS

C. pneumoniae EBs activate p44/p42 MAPK in human fibroblast cells via recognition of a putative chlamydial protein

The immunoblotting analysis demonstrated that purified C. pneumoniae EBs up-regulate the phosphorylation of p44/p42 MAPK in P-742 cells in a time-dependent manner (Fig. 1). The kinetics of p44/p42 MAPK activation revealed a peak at 5–15 min. Pretreatment of P-742 cells with the specific MAPK kinase 1/2 inhibitor PD98059 was shown to reduce C. pneumoniae-induced MAPK phosphorylation (lane 4) (Fig. 2). Stimulation with LPS-Re (lane 6, Fig. 2), a LPS of Re chemotype closely related to the chlamydial LPS antigen (Brade et al., 1997), had no effect on MAPK phosphorylation.

**Fig. 1.** C. pneumoniae-induced p44/p42 MAPK activation in P-742 cells. Serum-deprived cells were stimulated with purified viable 10⁶ IFU C. pneumoniae ml⁻¹ (m.o.i. = 4) for different periods of time. C, Control with mock-stimulated cells. Equal amounts of cell lysates (40 μg protein per lane) were analysed by immunoblotting using an antibody specific for active phospho-p44/p42 MAPK (upper). The equal protein amounts were verified with replicate blots incubated with rabbit antibodies specific for ERK1/2 expression. The immunoblotting for each experiment was performed three times.

In vitro infection assay. C. pneumoniae infection assay was performed as described previously with some modifications (Haralambieva et al., 2002b). Confluent P-742 cell monolayers, grown as cover slip cultures, were treated for 1 h where indicated with 10 μM Rolipram or 10 μM KN-62 and inoculated with 10⁵ IFU C. pneumoniae ml⁻¹ without centrifugation. The plates were incubated for 2 h at 37°C, washed five times with prewarmed RPMI and overlaid with 1 ml RPMI without cycloheximide, containing kinase inhibitors where indicated. After 48 h incubation at 37°C in a CO₂ atmosphere, the monolayers were fixed in methanol and stained for immunofluorescence with a genus-specific monoclonal antibody. The IFU were counted in 100 fields at a magnification of ×400 and the results were presented as percentage infectivity in IFU in comparison with the control IFU (100%).
Demonstrated the expression of TLR2 and TLR4 in P-742 cells via recognition of a chlamydial protein, and MAPK kinase inhibitor reduces C. pneumoniae-induced p44/p42 MAPK activation. Serum-deprived P-742 cells were mock-stimulated (lane 1) or stimulated for 10 min with 10^6 IFU purified C. pneumoniae ml^-1 (m.o.i. = 4), viable (lane 2) or heat-inactivated at 100 °C for 20 min (lane 5). In lane 6 cells were stimulated with 10 μg LPS-Re. In lanes 3 and 4 cells were treated with 50 μM PD98059 and the cells in lane 4 were stimulated with C. pneumoniae EBs. Equal amounts of cell lysates (40 μg protein per lane) were analysed by immunoblotting using an antibody specific for active phospho-p44/p42 MAPK (upper). The equal protein amounts were verified with replicate blots incubated with rabbit antibodies specific for ERK1/2 expression (lower). The immunoblotting experiments were performed three times with the same results.

Similarly, C. pneumoniae-induced MAPK activation was abolished by heating of EBs to 100 °C for 20 min (lane 5, Fig. 2), destroying the activity of most proteins, but not affecting LPS activity (Rietschel, 1984; Sasu et al., 2001).

**Cross-talk with other signalling pathways**

To elucidate the cross-talk with other signalling pathways, we assessed the effect of different kinase inhibitors on C. pneumoniae-induced MAPK activation. It was found that the selective PKC inhibitors Gö6976 and Gö6983, as well as the PKA inhibitor H-89, had no effect on C. pneumoniae-induced MAPK phosphorylation. In contrast, pretreatment of P-742 cells with the CaM kinase inhibitor KN-62 (lane 4) or with PDE 4 inhibitor Rolipram (lane 6) clearly enhanced C. pneumoniae-induced MAPK activation with little or no effect of the inhibitors on the basal activity (lanes 3 and 5, Fig. 3). MAPK phosphorylation triggered by the micro-organism was more pronounced in the presence of the two inhibitors. To ensure reliable quantitative assessment of results the immunoblotting experiments were performed three times with equal results, each time with a replicate blot for ERK1/2 expression to demonstrate equal protein amounts.

**C. pneumoniae activates p44/p42 MAPK in human fibroblast cells via TLR4**

To reveal the receptor mechanism of C. pneumoniae-stimulated MAPK activation in human fibroblast cells we first demonstrated the expression of TLR2 and TLR4 in P-742 cells by immunoblotting. Pretreatment of the cells with specific anti-TLR4 antibody (lane 6) was demonstrated to abolish C. pneumoniae-induced MAPK phosphorylation (lane 2), whereas an anti-TLR2 antibody had no or little effect (lane 4) (Fig. 4). The immunoblots were repeated three times with replicate blots for ERK1/2 expression.

**KN-62 and Rolipram attenuate C. pneumoniae infectivity in P-742 cells**

The effect of pharmacological enhancement of C. pneumoniae-induced MAPK phosphorylation on infectivity was evaluated by means of comparative in vitro infection assays. In parallel experiments with untreated and treated (with 10 μM KN-62 or 10 μM Rolipram) P-742 cells, it was found that KN-62 reduced C. pneumoniae infectivity 6.6-fold in vitro. The same effect was demonstrated with Rolipram, where a 5.5-fold decrease of infectivity was observed in comparison with the control cells (Fig. 5). The inhibitors had no effect on the appearance of the inclusions, which looked normal, just smaller in size, compared to the untreated culture.

**DISCUSSION**

C. pneumoniae is recognized as a significant cause of disease in the respiratory tract with most adults experiencing several infections over the course of a lifetime (Kauppinen & Saikku, 0.01)
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Fig. 4. C. pneumoniae activates p44/p42 MAPK in human fibroblasts via TLR4. Serum-deprived P-742 cells were left untreated or treated for 1 h prior to stimulation with anti-TLR2 antibody (lane 3 and 4) or with anti-TLR4 antibody (lane 5 and 6). Lane 1 represents a control with mock-stimulated P-742 cells. Cells were stimulated for 10 min with purified viable 10⁵ IFU C. pneumoniae ml⁻¹ (lanes 2, 4 and 6) and equal amounts of cell lysates were analysed by immunoblotting using an antibody specific for active phospho-p44/p42 MAPK (upper). The equal protein amounts were verified with replicate blots incubated with an antibody specific for ERK1/2 expression (lower). The immunoblotting experiments were performed three times with the same results.

Fig. 5. Effect of kinase inhibitors KN-62 and Rolipram on C. pneumoniae infectivity in vitro. P-742 cells were treated for 1 h with 10 μM KN-62 or with 10 μM Rolipram and then inoculated with 10⁵ IFU C. pneumoniae ml⁻¹ without centrifugation and cycloheximide. After 48 h incubation cell monolayers were stained for immunofluorescence with a genus-specific monoclonal antibody. The IFU were counted in 100 fields at a magnification of ×400 and the results are presented as percentage infectivity in IFU in comparison with the control IFU (100%) (P < 0.001). The presented results are means of three independent experiments with three replicates each.

1995). Infection with this micro-organism has also been associated with chronic inflammatory diseases such as asthma (Hahn et al., 1991) and atherosclerosis (Saikku et al., 1988). There is an increasing interest in the possibility that chronic C. pneumoniae infection might be involved in atherogenesis and, possibly, in plaque instability and acute coronary syndromes (Capron, 1996; Danesh et al., 1997; Libby et al., 1997; Kol & Libby, 1998). However, the molecular mechanisms by which C. pneumoniae might contribute to atheroma formation and lesional complications still remain unclear. This has become the focus of increased research effort, most of which involves elucidation of the interaction of the micro-organism with cells relevant to atherogenesis like macrophages, monocytes, smooth muscle cells and endothelial cells. In this regard little is known about human fibroblasts, which are a major cell population in chronic inflammation, contributing to plaque formation and evolution. Our previous work established the signalling mechanisms triggered by C. pneumoniae in mouse connective tissue fibroblasts L-929 (Haralambieva et al., 2002a). This study aimed to demonstrate C. pneumoniae-induced signal transduction pathways in human lung-derived fibroblasts and identify the engaged receptor mechanisms.

The MAPK cascade is extremely important for the regulation of gene expression in response to extracellular stimulation signals and subsequent activation of cell proliferation (Chang & Karin, 2001). The p44/p42 group of MAPKs is a central component of signalling via growth factors and other extracellular signals. Data from the literature demonstrate the signalling of C. pneumoniae and particular chlamydial components in different cell systems. C. pneumoniae-infected endothelial cells have been shown to express up-regulated phosphorylation of p44/p42 MAPK (Krüll et al., 1999). C. pneumoniae and chlamydial HSP60 were established as potent inducers of proliferation and MAPK activation in human smooth muscle cells (Sasu et al., 2001). C. pneumoniae was also demonstrated to induce tissue factor expression in mouse macrophages via activation of Egr-1 and the MEK-ERK1/2 pathway (Bea et al., 2003).

Our previous results in murine fibroblasts showed C. pneumoniae-stimulated p44/p42 MAPK phosphorylation with a peak at 30 min (Haralambieva et al., 2002a). The findings of this study established rapid C. pneumoniae-induced activation of p44/p42 MAPK in human lung-derived fibroblasts in a time-dependent manner with a peak at 5–15 min. The specific MAPK kinase 1/2 inhibitor PD98059 was demonstrated to reduce C. pneumoniae-induced MAPK phosphorylation. The principal chlamydial components that can trigger cell activation and atherogenesis include LPS, major outer-membrane protein, HSP and polymorphic membrane proteins (Kalayoglu et al., 2000; Niessner et al., 2003). Stimulation with heat-inactivated EBs (100 °C, 20 min), a treatment destroying most proteins but not LPS (Rietschel, 1984; Sasu et al., 2001), or stimulation with LPS-Te, a lipopolysaccharide of Te chemotype, closely related to the chlamydial LPS antigen (Brade et al., 1997), had no effect on MAPK phosphorylation in P-742 cells. The same
was found also in murine fibroblasts (Haralambieva et al., 2002a). These findings exclude the possibility of LPS involvement in MAPK activation and are consistent with the role of a putative heat-labile chlamydial protein.

Further, we tried to dissect the cross-talk between the MAPK cascade and other signalling pathways like PKC, PKA, CaM kinase, etc. PKC and PKA inhibition could lead to different effects on stimulated MAPK activity (Mitev et al., 1995; Le Panse et al., 1996). Our results established that *C. pneumoniae*-induced MAPK phosphorylation in human fibroblasts P-742 was PKC and PKA independent. However, the CaM kinase cascade and PDE 4 seemed to be involved in MAPK activation, since their inhibitors KN-62 and Rolipram enhanced *C. pneumoniae*-stimulated MAPK phosphorylation. Different reports from the literature tried to shed light on the importance of Ca$^{2+}$ and CaM kinases in diverse cellular responses. In rat smooth muscle cells, CaM kinase II was reported to act as an upstream activator of MAPK and its inhibition abolished MAPK activity (Abraham et al., 1997). The results of our study suggest a CaM-kinase-dependent mechanism of negative control on *C. pneumoniae*-stimulated MAPK activation in human fibroblasts, since CaM kinase inhibition promoted enhanced MAPK phosphorylation. The same mechanism was established in our previous work with murine fibroblasts (Haralambieva et al., 2002a). In addition, our findings in human lung fibroblasts demonstrated enhanced *C. pneumoniae*-induced MAPK activation upon treatment with Rolipram, a selective PDE 4 inhibitor and a major candidate for anti-asthmatic therapeutic agent (Banner et al., 1996). This observation could be of importance bearing in mind the possible association of *C. pneumoniae* infection with the pathogenesis of asthma as a chronic inflammatory disease (Hahn et al., 1991).

Recent studies have documented the role of the transmembrane Toll-like receptors in cellular activation by microbial pathogens (Means et al., 2000a). Toll-like receptors are recognized as the sensors of the innate immune system, linking the extracellular compartment, where contact with and recognition of microbial pathogens occurs, and the intracellular compartment, where signalling cascades leading to cellular responses are initiated (Vasselon & Detmers, 2002). Microbial components may interact with the leucine-rich extracellular domain of TLRs and subsequently activate multiple signalling pathways. Bacterial LPS-induced activation of NF-$\kappa$B and p44/p42 MAPK has been extensively studied, and is now known to involve TLR4 (Chow et al., 1999; Yang et al., 2000). Chlamydial HSP60 was demonstrated to activate macrophages and endothelial cells through the innate immune receptor complex TLR4–MD2 (Bulut et al., 2002). The authors established that both anti-TLR4 antibody and non-signalling TLR4 constructs, acting as dominant negative forms, were able to block HSP60-induced cellular activation. The predominant role of TLR2 versus TLR4 was reported in *C. pneumoniae*-stimulated activation of dendritic cells (Prebeck et al., 2001). In human smooth muscle cells, *C. pneumoniae* and chlamydial HSP60 were shown to induce proliferation via TLR4 and p44/p42 MAPK (Sasu et al., 2001), and the TLR4 antagonist RSLA (diphosphoryl lipid A from *Rhodobacter sphaeroides*) was demonstrated to inhibit cell proliferation and activation. The inhibitory effect of specific anti-CD14 and anti-TLR antibodies on stimulated cellular activation and cytokine production was successfully used by different authors (Kol et al., 2000; Bulut et al., 2002) to elucidate the receptor mechanisms involved. In this respect we have explored the effect of specific anti-TLR antibodies on *C. pneumoniae*-induced p44/p42 MAPK phosphorylation. The results of our study clearly demonstrated the blocking effect of the specific anti-TLR4 antibody (but not of anti-TLR2 antibody) on stimulated MAPK activation in three independent immunoblotting experiments. Our findings from the blocking studies are consistent with the role of TLR4 as a mediating receptor in *C. pneumoniae*-induced p44/p42 MAPK activation of human fibroblasts. However, further investigations in TLR-deficient cells such as human and mouse fibroblasts, transiently transfected with non-signalling TLR constructs, would definitely establish the role of TLR4 in *C. pneumoniae*-triggered signalling in fibroblasts.

The essential role of MAPK in cell protection against chlamydial infection has been demonstrated in human fibroblasts. In this study, the kinase inhibitors KN-62 and Rolipram, which enhanced the stimulated MAPK activation, were shown to reduce *C. pneumoniae* infectivity in vitro. The results support the protective role of MAPK in fibroblast cells and suggest novel therapeutic approaches to control chlamydial infection.

In conclusion, our results demonstrate the signalling mechanism of the respiratory pathogen *C. pneumoniae* in lung-derived fibroblasts of human origin, elucidating the receptors involved. The findings indicate that *C. pneumoniae* triggers rapid TLR4-mediated p44/p42 MAPK activation in human fibroblasts and chemical enhancement of MAPK phosphorylation modulates in vitro infection at the molecular level.

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