Chlamydia pneumoniae Induces Tissue Factor Expression in Mouse Macrophages via Activation of Egr-1 and the MEK-ERK1/2 Pathway

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Abstract—Recent studies have suggested that infection with Chlamydia pneumoniae (C pneumoniae) may contribute to the instability of atherosclerotic plaques and thrombosis and is associated with acute coronary events. Tissue factor (TF), a potent prothrombotic molecule, is expressed by macrophages and other cell types within atherosclerotic lesions and plays an essential role in thrombus formation after plaque rupture. Therefore the effects of C pneumoniae on induction of TF expression in macrophages were investigated. Infection of RAW mouse macrophages with C pneumoniae induced a time-dependent increase in procoagulant activity, expression of TF protein, and TF mRNA. C pneumoniae infection stimulated increased binding of nuclear proteins to the consensus DNA sequence for Egr-1, a key response element within the TF promoter, and increased the expression of Egr-1 protein. Transient transfections of RAW cells with mutated TF promoter constructs showed that the Egr-1 binding region is an important transcriptional regulator of C pneumoniae–induced TF expression. Furthermore, C pneumoniae–stimulated phosphorylation of ERK1/2 and Elk-1 and pharmacological inhibition of mitogen-activated protein kinase activity reduced the expression of TF and Egr-1. Antibody and polymyxin B blocking of the Toll-like receptor 4 (TLR4) partially reduced the C pneumoniae–induced expression of TF and Egr-1. In conclusion, the C pneumoniae–induced increase in TF expression in macrophages is mediated in part by Egr-1, signaling through TLR4, and activation of the MEK-ERK1/2 pathway. (Circ Res. 2003;92: 394-401.)

Key Words: arteriosclerosis ■ Chlamydia pneumoniae ■ tissue factor ■ signal transduction

Epidemiological studies suggest that infection with Chlamydia pneumoniae (C pneumoniae) is an independent risk factor for cardiovascular disease. C pneumoniae can be detected in ≈50% of atheromatous lesions in humans and is able to infect and multiply in several cell types present in the vascular wall.1, 2 Moreover, experimental studies in animal models have demonstrated increased progression of atherosclerotic lesions after infection with C pneumoniae.3, 4 Recent clinical studies have suggested that infection with C pneumoniae is associated with increased plaque instability and thrombogenesis during acute coronary events.5, 6 Tissue factor (TF), a potent prothrombotic molecule, is thought to play an important role in stimulating thrombus formation after plaque rupture.7, 8 In vitro studies have demonstrated increased expression and activation of TF by a variety of stimuli, such as lipopolysaccharide (LPS), oxidized low-density lipoprotein (oxLDL), shear stress, tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and CD40 ligand.9, 10, 11 In addition, infection with C pneumoniae has been reported to increase expression of TF in endothelial cells and smooth muscle cells.12 However, the effects of C pneumoniae infection on TF expression in macrophages remain unknown. This is despite the fact that macrophages disseminate C pneumoniae infection from the lungs, have been implicated as the main source of intraplaque TF, and are thought to play a key role in destabilizing atherosclerotic plaques and in the formation of thrombotic occlusions after plaque rupture.13, 14, 15

The transcriptional regulation of the TF gene varies depending on the cell type and stimulus. Functional analyses of the TF promoter have identified putative AP-1, nuclear factor-κB (NF-κB), Sp1, and Egr-1 binding sites, 11, 14, 15 and C pneumoniae has been reported to increase activation of the NF-κB/Rel family and AP-1 in various cell types.16, 17, 18 Recent studies also suggest that Egr-1 plays a major role in TF gene expression in monocytic cells in response to inflammatory stimuli.19 However, the effect of C pneumoniae infection on Egr-1 expression and activity has not yet been...
reported. Thus, the objective of the present study was to investigate whether *C pneumoniae* infection induces TF expression in macrophages via increased activation of Egr-1. Furthermore, the role of the Toll-like receptor 4 (TLR4) that binds LPS and the downstream MEK-ERK1/2 pathway in *C pneumoniae*-induced expression of TF and Egr-1 was evaluated, as signal transduction through the MEK-ERK1/2 pathway has been reported to activate Egr-1.11

**Materials and Methods**

**Cell Culture and Preparation of *C pneumoniae***

RAW 264.7 cells, a murine macrophage cell line (ATCC, Manassas, Va), were grown in DMEM (Gibco BRL) containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. *C pneumoniae* (strain AR-39) was grown in HL cells and purified by density gradient centrifugation using diatrizoate meglumine (Hypaque-76; Winthrop-Breon Laboratories). The purified organisms were resuspended in sucrose phosphate glutamic acid and frozen at −70°C until use. A multiplicity of infection of 15 was used for infection of the RAW cells in all experiments. As a control for some experiments, *C pneumoniae* were inactivated by heat (30 minutes at 56°C). The MEK inhibitor PD98059 was obtained from Calbiochem, and polymyxin B (2 μg/mL, 30 minutes at room temperature) was obtained from Sigma.

**Isolation of RNA and Real-Time RT-PCR**

RAW cells were seeded at a density of 5×10⁵ cells per well in 6-well culture plates 1 day before the experiment. Total RNA was extracted from the cells using the Tripure reagent (Boehringer Mannheim) according to the manufacturer’s protocol. Real-time quantitative RT-PCR (Taqman ABI Prism 7700 Sequence Detection System, PE Biosystems) was performed using 1 μg of total RNA to determine relative expression levels of TF and β-actin. The primer sequences for TF and β-actin used were as follows: for TF forward, 5'-CACATCATCTTTGGGAGCAGTG-3' and reverse, 5'-CCCTAAGGGGCAACCTGGAA-3' and reverse, 5'-ACGACCAAGGCATACAGGGA-3'. The transcription for the constitutive gene product β-actin was used for data normalization.

**Procoagulant Activity Assay**

RAW cells (1×10⁶) were solubilized with 15 mmol/L octyl-p-glucopyranoside (Sigma) at 37°C for 15 minutes. Procoagulant activity levels were evaluated using a one-step clotting assay as previously described.11

**Plasmids and Transient Transfections**

The wild-type rat TF promoter (−143) and a construct containing a mutation of the Egr-1 (Egr-1−) site have been described elsewhere.12 For transfections, cells were seeded at a density of 5×10⁵ cells per well in a 6-well culture plate the day before the experiment. Cells were transfected with 2.5 μg DNA/well for 5 hours using Lipofectamine reagent (Gibco) according to the manufacturer’s protocol. All samples were cotransfected with equal amounts of a pRL-TK construct encoding Renilla luciferase (Promega) to compensate for variation in transfection efficiencies (−10%).Twenty-four hours after transfection, cells were infected with *C pneumoniae* for 12 hours. Cells were harvested and luciferase assays were performed using the Dual Luciferase Reporter System (Promega) according to the manufacturer’s protocol.

**Preparation of Nuclear Extracts**

Nuclear extracts were isolated using the method of Hoppe-Seyler et al.22 Briefly, 4×10⁶ cells were lysed directly on the culture dish in 1.0 mL cold lysis buffer (0.6% NP40, 0.15 mol/L NaCl, 10 mmol/L Tris [pH 7.9], and 1 mmol/L EDTA), scraped, and pelleted. The nuclear proteins were extracted into 50 μL cold extraction buffer containing 10 mmol/L HEPES [pH 7.9], 0.1 mmol/L EGTA, 0.1 mmol/L EDTA, 1.5 mmol/L MgCl₂, 420 mmol/L NaCl, 25% glycerol, and a protease inhibitor cocktail of pepstatin A, E-64, bestatin, leupeptin, and aprotinin (Sigma). Nuclear extracts were stored at −80°C until analysis. The protein concentration was measured using the Bradford protein dye reagent (Bio-Rad).

**Electrophoretic Mobility Shift Assay (EMSA)**

Double-stranded oligonucleotides containing the consensus binding site for Egr-1, Sp1 (Santa Cruz Biotechnology), and three regions of the serum response region of the rat TF promoter12 were radiolabeled with γ-³²P-ATP using T-4 polynucleotide kinase (Promega). The labeled oligonucleotides were incubated with 5 μg of nuclear proteins and loaded on a 4% nondenaturing acrylamide gel for separation from the unbound oligonucleotides according to the manufacturer’s manual (Promega). For competition and antibody supershift assays, unlabeled oligonucleotides or a rabbit polyclonal antibody against Egr-1 (C-19, Santa Cruz) were added subsequent to the radiolabeled oligonucleotides. Gels were analyzed by phosphorimaging (Cyclone, Packard Instruments).

**Western Blotting**

Cell lysates (27 μg/lane) or nuclear extracts (15 μg/lane) were separated by SDS-PAGE using a 12% separating gel and transferred to a nitrocellulose membrane for immunoblotting. The blot was blocked overnight at 4°C in TBST-5% nonfat milk, incubated with the primary antibody for 1 hour at room temperature, and washed with TBST. The blot was then incubated with the secondary antibody, washed in TBST, visualized with ECL, and quantified by NIH Image 1.62. The antibodies utilized for these studies included goat anti-human TF (American Diagnostica), goat anti-α-actin, rabbit anti-Egr-1 (Santa Cruz), rabbit antimouse MAP44/42 mitogen-activated protein (MAP) kinase, and anti-phospho-p44/42 MAP kinase (Cell Signaling), and rabbit anti-p-Erk, and anti-Erk (Santa Cruz).

**Blocking of TLR4**

RAW mouse macrophages were pretreated for 30 minutes at 37°C with rat anti-mouse TLR4/MD2 (20 μg/mL, functional grade clone MTS551) or a rat IgG2a isotype control (eBioscience). Cells were harvested at 2 hours after infection for extraction of nuclear proteins and at 3 hours after infection for RNA extraction.

**Statistical Analysis**

All data were expressed as mean±SEM. Significant differences between means were determined by using the Student’s two-tailed t test. All experiments were performed at least three times and representative results are shown.

**Results**

*C pneumoniae* Infection Increases TF mRNA, Protein, and Activity in Macrophages

Mouse RAW cells were infected with *C pneumoniae*, and the resulting changes in the level of procoagulant activity, TF protein, and TF mRNA were determined using a clotting assay, Western blot analysis, and real-time quantitative RT-PCR, respectively. Infection with *C pneumoniae* increased procoagulant activity by 6 hours after infection and was maximum at 12 hours after infection (Figure 1A). *C pneumoniae* induced an increase in TF protein levels by 2.2-fold with maximal protein synthesis observed 12 hours after infection. In contrast, the content of TF protein was very low in uninfected cells. Reprobing of the Western blot for α-actin
demonstrated equal protein loading (Figure 1B). TF mRNA levels were increased ≈12-fold, with maximal induction observed at 3 hours after infection (Figure 1C).

**C pneumoniae** Induces Binding of Nuclear Factors to the Egr-1 Consensus Binding Sequence and Increases Nuclear Egr-1 Protein Levels

EMSAs were conducted with RAW cell nuclear extracts collected at various time points after infection. Infection of the RAW cells with *C pneumoniae* induced binding to the Egr-1 consensus sequence, with maximal binding occurring within 2 to 4 hours of infection (Figure 2A). Nuclear factor activation and binding were diminished by 6 to 9 hours and were not detectable during most of the 72-hour growth cycle of *C pneumoniae* (data not shown). The specificity of the binding was demonstrated after addition of a 100-fold excess of unlabeled Egr-1 oligonucleotide (Figure 2A, lane 7) and by the lack of nuclear factor binding to a 32P-labeled mutant Egr-1 oligonucleotide differing from the wild-type consensus sequence by two base pairs (Figure 2A, lane 9). Furthermore, addition of an anti–Egr-1 antibody caused a supershift establishing the presence of the transcription factor in the binding complex (Figure 2A, lane 8). Binding of nuclear proteins to the consensus sequence for Sp1 demonstrated a constitutive binding pattern and was not affected by infection with *C pneumoniae* (Figure 2B).

Western blot analysis with an anti–Egr-1 antibody was used to determine the influence of *C pneumoniae* infection on the levels of Egr-1 protein in nuclear extracts from infected RAW cells. *C pneumoniae* infection induces a transient increase in Egr-1 protein levels in the nuclei of infected RAW cells with maximum Egr-1 protein levels observed at 1 to 2 hours after infection (Figure 2C).

**C pneumoniae** Selectively Induces Binding of Nuclear Proteins to the Egr-1 Binding Site of the Serum Response Region (SRR) Within the TF Promoter

Induction of the TF gene is mediated in part by a highly conserved proximal region in the rat TF promoter (−143 to +14 bp relative to the start site of transcription) called the SRR. This promoter area is subdivided into three regions, RI, RII, and RIII, corresponding to the three Sp1 binding sites contained therein.12 The third region, RIII, also includes a 12-bp motif that contains a putative Egr-1 site overlapping the Sp1 binding site (Figure 3A). Therefore, EMSAs were performed with three different oligonucleotides representing the three different regions of the promoter as depicted in Figure 3A. Strong constitutive binding of nuclear factors to
RIII (Figure 3B) and RI and RII (data not shown) was observed with two apparent principal bands predominant. However, infection with C pneumoniae induced the appearance of a third band only in RIII that was competitively inhibited by the addition of excess unlabeled Egr-1 consensus oligonucleotide (Figure 3B, arrow, lanes 2 and 4). Excess unlabeled Egr-1 oligonucleotide had no effect on the constitutive protein binding to the two principal bands in any region (Figure 3B, lanes 3 and 4). In contrast, addition of excess unlabeled Sp1 consensus oligonucleotide strongly inhibited binding of nuclear factors at the two constitutive bands (Figure 3B, lanes 5 and 6) but had no effect on the C pneumoniae–induced third band appearing in RIII (Figure 3B, lane 6). These data are consistent with Sp1-mediated constitutive expression of the TF gene at all three regions and C pneumoniae–induced Egr-1–mediated expression at the overlapping Egr-1/Sp1 site in RIII.

Mutation of the Egr-1 Binding Site Inhibits Induction of Reporter Activity by C pneumoniae
RAW cells were transiently transfected with plasmids containing the wild-type TF promoter and with plasmids containing a mutation of the Egr-1 binding site for 24 hours. After transfection, cells were infected with C pneumoniae for 12 hours. Luciferase activity in cell lysates was determined. *P<0.05 vs infected wild-type TF promoter.

C pneumoniae Induces Phosphorylation of ERK1/2 and Elk-1
Phosphorylation of ERK1/2 was induced in a time-dependent manner after infection with C pneumoniae. Maximum phosphorylation of ERK1/2 was observed 30 minutes after infection with C pneumoniae (Figure 5A). Parallel to the phosphorylation of ERK1/2, transient phosphorylation of Elk-1 that peaked at 45 minutes after infection with C pneumoniae was also observed (Figure 5B). There were no increases in the nonphosphorylated forms of ERK1/2 or Elk-1 after infection with C pneumoniae (Figures 5A and 5B).

C pneumoniae Induction of TF Expression via Egr-1 Involves the MEK-ERK1/2 Kinase Pathway
RAW cells were pretreated with 25 μmol/L of the MEK-ERK1/2 inhibitor PD98059 for 60 minutes, and TF expression was determined by real-time RT-PCR after infection with C pneumoniae for 3 hours. The presence of PD98059 significantly inhibited the induction of TF expression by ~50%, suggesting that the MEK-ERK1/2 MAP kinase pathway plays a role in the induction of TF expression after infection with C pneumoniae (Figure 6A). The pretreatment of RAW cells with PD98059 before infection with C pneumoniae also inhibited the C pneumoniae–induced increase in TF expression by ~50%.

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Figure 4. Reporter construct analysis of the contribution of the Egr-1 binding site to TF expression. RAW cells were transiently transfected with plasmids containing the wild-type TF promoter and with plasmids containing a mutation of the Egr-1 binding site for 24 hours. After transfection, cells were infected with C pneumoniae for 12 hours. Luciferase activity in cell lysates was determined. *P<0.05 vs infected wild-type TF promoter.
Egr-1 protein and binding to DNA as evaluated by Western blot and EMSA, respectively (Figures 6B and 6C).

Both the LPS and Unknown Protein Components of C pneumoniae Contribute to TF Expression and Egr-1 Activation

Heat inactivation (56°C, 30 minutes) reduced, but did not abolish, the C pneumoniae induction of TF expression (borderline significance was obtained with triplicate determinations, \( P = 0.14 \), Figure 7A). Furthermore, inoculation of the RAW cells with heat-inactivated C pneumoniae also partly reduced the Egr-1 content in the nucleus as shown by Western blotting (Figure 7B). This suggests that a heat-insensitive component such as the LPS of C pneumoniae combines with an as yet unknown heat-sensitive chlamydial protein to induce TF expression and Egr-1 activation. This is further supported by the reduced TF expression (borderline significance was obtained with triplicate determinations, \( P = 0.07 \), Figure 7A) and Egr-1 activation observed after pretreatment of C pneumoniae with polymyxin B, a known inhibitor of LPS (Figure 7B) and by the partial reduction of expression observed after antibody blocking of the TLR4 (Figures 7C and 7D).

Discussion

Data from epidemiological, histopathological, and intervention studies suggest a role of C pneumoniae infection in the pathogenesis of the acute coronary syndrome.\(^8,9,23,24\) However, the cellular and molecular mechanisms by which C pneumoniae participates in the development, progression, and destabilization of atherosclerotic lesions remain to be elucidated. Several possible mechanisms include C pneumoniae–induced increases in macrophage accumulation of lipids,\(^20,25\) secretion of proinflammatory cytokines such as TNF-\(\alpha\), IL-6, interferon-\(\gamma\) (IFN-\(\gamma\)), and IL-1\(\beta\),\(^20,26,27\) increased expression of the adhesion molecules E-selectin, ICAM-1, and VCAM-1,\(^28\) and increased expression of matrix metalloproteinases.\(^29\)

The formation of thrombi after rupture of unstable atherosclerotic plaques leads to acute coronary events.\(^30\) Various components of atherosclerotic plaques contribute to the overall thrombogenicity of the plaque. In this regard, the exposure of TF and stimulation of the extrinsic pathway of coagulation are thought to be central to thrombus formation after rupture.\(^10\) Macrophages are one of the predominant cell types in unstable plaques and are the main source of intraplaque TF.\(^10\)
The LRR contains the promoter known as the LPS response region (LRR) and the bind to two response elements within the rat TF proximal regulators of TF gene expression. These transcription factors

Our data clearly demonstrate increased procoagulant activity, increased TF protein, and increased expression of TF mRNA in macrophages after infection with *C pneumoniae*. These data now provide a plausible mechanism for explaining how *C pneumoniae* infection can contribute to the acute coronary syndrome. However, demonstration of a direct association between macrophages expressing TF and the presence of *C pneumoniae* particles or antigen in atherosclerotic plaques from either humans or experimental animals has been problematic. This is likely due to the small number of *C pneumoniae* particles and the widespread distribution of TF observed within most lesions. This points to the fact that TF expression can be simultaneously induced by a variety of stimuli, such as other forms of LPS, oxLDL, cytokines, and CD40 ligand.11–14

Previous studies have identified the transcription factors NF-κB, AP-1, Sp1, and Egr-1 as important transcriptional regulators of TF gene expression. These transcription factors bind to two response elements within the rat TF proximal promoter known as the LPS response region (LRR) and the SRR.12,19 The LRR contains the cis-regulatory element for NF-κB and AP-1 binding on stimulation by inflammatory mediators such as LPS, TNF-α, or IL-1β.31,32 In contrast, the SRR mediates induction of TF expression by serum, lipoproteins, or shear stress12,13,32 and contains three Sp1 and one Egr-1 binding sites. However, recent studies have also demonstrated a role for Egr-1 in the expression of TF in response to inflammatory stimuli.11 In support, our data demonstrate that infection with *C pneumoniae* leads to increased TF mRNA and protein that is associated with increased binding of Egr-1 to the SRR of the TF promoter and to the consensus Egr-1 sequence. The specificity of the binding and its direct effect on TF expression were clearly verified using the mutated reporter construct. These data are in agreement with several other studies that have demonstrated increased binding to Egr-1 sites within the TF promoter after treatment with CD40 ligand, oxLDL, and hypoxia.12,14,33

Egr-1 is abundantly expressed in human atherosclerotic lesions, and its expression is induced in the aorta of LDL receptor–deficient mice after cholesterol feeding.12 Egr-1 also plays a role in the regulation of the expression of growth factors such as platelet-derived growth factor and basic fibroblast growth factor, cytokines such as TNF-α, and adhesion molecules such as ICAM.35 Thus, activation of Egr-1 in macrophages by *C pneumoniae* is likely to have a broader effect on the atherogenic process than simply increasing TF production and may help explain how *C pneumoniae* contributes to both the initiation and progression of atherosclerosis.

Figure 7. Induction of TF and Egr-1 expression is in part mediated by both the LPS and protein component of *C pneumoniae*. RAW cells were infected with *C pneumoniae* (CP) and *C pneumoniae* that was heat-inactivated (37°C, 30 minutes) or pretreated with polymyxin B (2 μg/mL, 30 minutes at room temperature). Total RNA was extracted 3 hours after infection, and TF mRNA levels were determined by real-time RT-PCR (A). Total RNA was extracted 3 hours after infection, and TF mRNA levels were determined by real-time RT-PCR. *P<0.05 vs Control-AB+CP, panel C). Nuclear extracts were harvested 2 hours after infection with *C pneumoniae*, and Egr-1 expression was determined by Western blotting (B). RAW cells were pretreated for 30 minutes at 37°C with a blocking antibody against TLR4 or an irrelevant antibody of the same isotype as control. Total RNA was extracted 3 hours after infection, and TF mRNA levels were determined by real-time RT-PCR. °P<0.05 vs Control-AB+CP, panel C). Nuclear extracts were harvested 2 hours after infection with *C pneumoniae*, and Egr-1 expression was determined by Western blotting (D).

Our data clearly demonstrate increased procoagulant activity, increased TF protein, and increased expression of TF mRNA in macrophages after infection with *C pneumoniae*. This is consistent with a previous study by Guha et al,11 showing that in human monocytes treated with LPS derived from *E coli*, there is activation of the MEK-ERK1/2 kinase pathway, increased phosphorylation of Elk-1, and increased expression of Egr-1, TF, and TNF-α. This is consistent with a previous study by Guha et al,11 showing that in human monocytes treated with LPS derived from *E coli*, there is activation of the MEK-ERK1/2 kinase pathway, increased phosphorylation of Elk-1, and increased expression of Egr-1, TF, and TNF-α.

It is currently unclear whether the *C pneumoniae*–induced activation of the MEK-ERK1/2 kinase pathway and increases in TF expression and Egr-1 activation are in response to *C pneumoniae* LPS and/or to chlamydial proteins such as heat shock protein 60 (Hsp60). As noted, *E coli* LPS is a major inducer of TF expression,11 and the stimulatory effects of *E coli* LPS on macrophages are currently thought to be dependent entirely on binding of the LPS to the TLR4.36 However, LPS from *C pneumoniae* is immunologically different from that of *E coli*.38 Thus, it is possible that the LPS from *C pneumoniae* does not mediate its effects via the same mechanisms as LPS from *E coli*. Our data showing that neither heat treatment nor an antibody to the TLR4 entirely abolishes the *C pneumoniae*–induced increase in TF expression suggest that unlike *E coli*, both the heat-insensitive LPS and some heat-sensitive protein of *C pneumoniae* combine to activate the macrophages via binding to both the TLR4 and...
potentially other sites. This was further supported by our observation that polymyxin B treatment of C pneumoniae also reduces TF and Egr-1 expression. It is also supported by studies showing that C pneumoniae Hsp60 can activate macrophages through binding to the TLR4 and that blocking antibodies against the TLR2 but not TLR4 or CD14 inhibit the C pneumoniae–induced increase in TNF-α expression in peripheral blood monocytes. Furthermore, macrophages from C3H/HeJ mice that have a defect in the TLR4 exhibit no reduction in their capacity to express TNF–H9251 phages from C3H/HeJ mice that have a defect in the TLR4 also been reported for the C pneumoniae.  

In conclusion, the present data demonstrate for the first time that C pneumoniae induces the expression of TF in macrophages and that this induction involves Egr-1 and the MEK-ERK1/2 kinase pathway. We have also shown that induction of TF expression is in part dependent on binding of C pneumoniae components to the TLR4. These observations should help to explain how C pneumoniae infection may contribute to the acute coronary syndrome.

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