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.1–3 Moreover, experimental studies in animal models have demonstrated increased progression of atherosclerotic lesions after infection with C pneumoniae.4–7 Recent clinical studies have suggested that infection with C pneumoniae is associated with increased plaque instability and thrombogenesis during acute coronary events.8,9 Tissue factor (TF), a potent prothrombotic molecule, is thought to play an important role in stimulating thrombus formation after plaque rupture.10

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.11–14 In addition, infection with C pneumoniae has been reported to increase expression of TF in endothelial cells and smooth muscle cells.15 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.16–18

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,11,14,19 and C pneumoniae has been reported to increase activation of the NF-κB/Rel family and AP-1 in various cell types.15,20,21 Recent studies also suggest that Egr-1 plays a major role in TF gene expression in monocytic cells in response to inflammatory stimuli.11 However, the effect of C pneumoniae infection on Egr-1 expression and activity has not yet been
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 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.

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 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 anti–p44/42 mitogen-activated protein (MAP) kinase, and anti–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 MTS510) 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.
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 (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. 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.

**Mutatin of the Egr-1 Binding Site Inhibits Induction of Reporter Activity by *C pneumoniae***

RAW cells were transiently transfected with plasmids containing the TF promoter SRR linked to a luciferase reporter. The reporter constructs contained either a wild-type Egr-1 binding site (TF-143) or a mutated Egr-1 binding site (TF-143 Egr-1 m ). *C pneumoniae* infection of the RAW cells that had been transfected with the promoter construct containing the wild-type Egr-1 binding site increased luciferase reporter activity by 3.4-fold over nontreated cells (Figure 4). In contrast, infection of the cells that had been transfected with the mutated Egr-1 site induced ~50% less luciferase activity than the cells transfected with the wild-type construct (Figure 4), indicating that Egr-1 plays an important role in regulating TF gene expression in response to infection with *C pneumoniae*.

**C pneumo**niae 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
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. 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, secretion of proinflammatory cytokines such as TNF-α, IL-6, interferon-γ (IFN-γ), and IL-1β, increased expression of the adhesion molecules E-selectin, ICAM-1, and VCAM-1, and increased expression of matrix metalloproteinases.

The formation of thrombi after rupture of unstable atherosclerotic plaques leads to acute coronary events. 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. Macrophages are one of the predominant cell types in unstable plaques and are the main source of intraplaque TF.
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.

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. 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. In contrast, the SRR mediates induction of TF expression by serum, lipoproteins, or shear stress 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. 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. 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. 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. 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.

Binding of bacterial products to toll-like receptors (TLRs) can activate the MAP kinase pathway. The activation of the MEK-ERK1/2 kinase in response to C. pneumoniae infection has been demonstrated in endothelial cells and smooth muscle cells but little is known about the activation of the ERK1/2 kinase in macrophages after infection with C. pneumoniae. Our data demonstrate that the MEK-ERK1/2 kinase pathway and Elk-1 in macrophages are activated by treatment with C. pneumoniae. Furthermore, we have shown that inhibition of MEK-ERK1/2 reduces expression of TF and Egr-1. This is consistent with a previous study by Guha et al., 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, 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. However, LPS from C. pneumoniae is immunologically different from that of E. coli. 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 also exhibit no reduction in their capacity to express TNF-α in response to *C. pneumoniae*. Involvement of the TLR2 has also been reported for the *C. pneumoniae*-induced increase in TNF-α expression by dendritic cells.

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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**References**


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