# *Chlamydophila pneumoniae* Induces ICAM-1 Expression in Human Aortic Endothelial Cells via Protein Kinase C–Dependent Activation of Nuclear Factor-κB

Silvana A. Vielma, Gregor Krings, Maria F. Lopes-Virella

***Abstract***—*Chlamydophila pneumoniae* has an epidemiological link with atherosclerosis and acute cardiovascular events. One mechanism that may explain such a link is the increased expression of intracellular adhesion molecule-1 (ICAM-1) in *C pneumoniae*–infected endothelial cells. Upregulation of ICAM-1 by *C pneumoniae* is well recognized and has been extensively studied, but the signaling pathways involved are not yet defined. Because upregulation of ICAM-1 by cytokines and other stimuli has been shown to be mediated by either mitogen-activated protein kinase, protein kinase C (PKC), or nuclear factor-κB (NF-κB) pathways, we examined whether these pathways were involved in the ICAM-1 upregulation induced by *C pneumoniae*. Our data show a time-dependent phosphorylation of p44/p42 and SAPK/JNK pathways in *C pneumoniae*–infected cells. However, inhibition of the classic mitogen-activated protein kinase pathway using the PD98059 and U0126 inhibitors and inhibition of SAPK/JNK pathway did not suppress *C pneumoniae*–induced ICAM-1 expression. *C pneumoniae* also activates the NF-κB pathway at 30 minutes after infection. Treatment of human aortic endothelial cells (HAECs) with the NF-κB inhibitors BAY117085 and caffeic acid phenethyl ester led to a concentration-dependent inhibition of *C pneumoniae*–induced ICAM-1 upregulation. Finally, *C pneumoniae*–infected HAECs show membrane translocation of total PKC 30 minutes after cell infection. Calphostin C, a general PKC inhibitor, blocked both *C pneumoniae*–induced ICAM-1 expression and *C pneumoniae*–induced NF-κB translocation. In conclusion, we demonstrated that *C pneumoniae*–induced ICAM-1 expression in HAECs requires NF-κB and PKC activation and that NF-κB activation is PKC dependent. **(*Circ Res*. 2003;92:1130-1137.)**

**Key Words:** *Chlamydophila pneumoniae* ■ intercellular adhesion molecule-1 ■ nuclear factor-κB ■ protein kinase C ■ mitogen-activated protein kinase pathway

Intercellular adhesion molecule-1 (ICAM-1), a member of the immunoglobulin supergene family, plays a role in immune and inflammatory responses[1] because of its critical role in mediating monocyte adhesion to the endothelium[2] as well as transmigration of leukocytes. It also serves as a coactivation signal in T cell activation[3] and in cell-matrix adhesive interactions.[4] ICAM-1 expression is upregulated by several inflammatory mediators, such as cytokines (interleukin-1β [IL-1β], tumor necrosis factor [TNF], and interferon-γ),[5] oxidant stress,[6] shear stress, and low-density lipoproteins (native LDL and oxidize LDL).[7,8] In addition, ICAM-1 serves as a receptor for rhinoviruses,[9] and it is upregulated by a variety of microorganisms, including *Chlamydophila pneumoniae*.[10] ICAM-1 is minimally expressed in normal endothelium; however, in the earlier stages of atherosclerosis, significant upregulation of ICAM-1 expression is usually observed.[11]

*C pneumoniae* is an important respiratory pathogen associated with pneumonia, pharyngitis, bronchitis, and sinusitis.[11] In the last decade, an association of *C pneumoniae* with atherosclerosis and coronary artery disease has been described by numerous investigators.[12–15] *C pneumoniae* is able to replicate in macrophages, endothelial cells, and smooth muscle cells,[16–18] the 3 major cell types present in atherosclerotic lesions. Infection of human endothelial cells with *C pneumoniae* has been shown to upregulate the expression of adhesion molecules, cytokines, and growth factors.[19–21] It has been also shown that *C pneumoniae*, by leading to increased expression of cell adhesion molecules,[10,22] induces transendothelial migration of neutrophils and monocytes[22] and promotes activation of proteins with procoagulant activity.[23] Regardless of the extensive literature on *C pneumoniae*–induced upregulation of ICAM-1, very little is known about the signal transduction pathways involved in the process.

Therefore, we decided to investigate the signaling pathways involved in *C pneumoniae*–induced ICAM-1 upregulation in human aortic endothelial cells (HAECs) to identify possible therapeutic targets to prevent ICAM-1 upregulation.

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Because upregulation of ICAM-1 by cytokines and other stimuli has been shown to be mediated by mitogen-activated protein kinase (MAPK), protein kinase C (PKC), or nuclear factor-κB (NF-κB) pathways,[4] we decided to investigate whether some or all of the above pathways were involved in the ICAM-1 upregulation induced by *C pneumoniae*.

## Materials and Methods

### Cell Cultures

Hep-2 cells (ATCC CCL23) were maintained in minimal essential medium (Sigma-Aldrich Co) containing Earle's salts (EMEM) following ATCC recommendations. Primary HAECs were cultured following manufacturer's recommendations (Cascade Biologics, Inc) and used in passages 2 to 6.

### *Chlamydophila pneumoniae* Propagation

*C pneumoniae* AR39 (ATCC 53592) was propagated in Hep-2 cell monolayers as described before.[24] After 72 hours of incubation, *C pneumoniae* was harvested by mechanical disruption, followed by a low-speed (250*g*) centrifugation. Elementary bodies present in the supernatant were pelleted at 30 000*g* (JA-20 rotor, Beckman Instruments, Inc) for 30 minutes. The pellets were suspended in 0.01 mol/L sodium phosphate (pH 7.2) containing 0.25 mol/L sucrose and 5 mmol/L L-glutamic acid (SPG)[25,26] and stored at −70°C. HAECs were infected as previously described.[24] In brief, HAECs grown in 6-well plates ($2.5\times10^5$ cells/well) were infected with 2 mL of medium containing 6 to $10\times10^5$ inclusion forming units (IFUs). HAECs grown in 100-mm dishes ($1.5\times10^6$ cells) were infected with 8 mL of medium containing $\approx16\times10^6$ IFU.

In some experiments, before addition to the cells, *C pneumoniae* was heat treated for 30 minutes at 95°C or UV irradiated for 30 minutes at 15 cm from the UV source ($1200\times100$ mJ/cm$^2$, Hoefer UVC500).[21] Because the *C pneumoniae* inoculum may contain remnants of HEp-2 cells, we used as controls mock-infected cells (HAECs treated with crude preparations of noninfected HEp-2 cells processed in the same manner as infected Hep-2 cells). HAEC monolayers grown in coverslips were used to monitor the course of infection by immunofluorescence staining using a genus-specific fluorescein isothiocyanate (FITC)-labeled monoclonal antibody (Pathfinder Chlamydia confirmation system; Bio-Rad). Titration of *C pneumoniae* in infected HAECs was assessed according to previously published methodology.[17]

### ICAM-1 Expression by Immunoblotting

HAECs were lysed with Triton X buffer (10 mmol/L HEPES, 200 mmol/L NaCl, 2 mmol/L $CaCl_2$, 2.5 mmol/L $MgCl_2$, 1.5% vol/vol Triton X) containing 100 μg/mL phenylmethylsulfonyl fluoride and 10 μg/mL leupeptin (Sigma-Aldrich Co). Cell lysates were centrifuged, and the protein concentration in the supernatant was determined using a BCA Protein Assay Reagent Kit (Pierce). Western blots were performed as described[27] using an anti-hICAM-1 monoclonal antibody diluted at 1:500 (Calbiochem-Novabiochem). A horseradish peroxidase–conjugated anti-mouse antibody (Calbiochem-Novabiochem) was used as secondary antibody. ICAM-1 was visualized after incubation of the membranes in Chemiluminescence Reagent Plus (Perkin Elmer Life Science, Inc) followed by exposure to X-ray film.

In some experiments, HAECs were incubated for 30 to 60 minutes with inhibitors of the several signaling pathways studied before *C pneumoniae* infection. As inhibitors for the MAPK pathways, the MEK1/2 inhibitor U0126 (Cell Signaling Technology), the MEK1 inhibitor PD98059, and the Jun N-terminal kinase (JNK) inhibitor SP600125 (Calbiochem-Novabiochem) were used. BAY11-7085 and caffeic acid phenethylester (CAPE) were used to inhibit the NF-κB pathway, and calphostin C, bisindolylmaleimide I, and Gö 6976 were used to inhibit PKC pathway. All of the above inhibitors were from Calbiochem-Novabiochem.

The toxicity to HAECs of all the inhibitors used was determined by cell morphology and trypan blue dye exclusion of trypsinized cells using different concentrations of the drugs. Concentrations not toxic to HAECs were chosen.

### Northern Blot Analysis

ICAM-1 cDNA (1 mg/mL) was generously provided by Dr Jeffrey Greve (Bayer Pharmaceutical Division, Berkeley, Calif). The cDNA probe was prepared by polymerase chain reaction (PCR) using a Qiagen Taq DNA Polymerase Kit (Qiagen). The following primers were used: 5′-GAGATCACCATGGAGCCAAT-3′/5′-GGG-CCTCACACTTCACTGTC-3′ (Sigma-Genosys).

Total cellular RNA was isolated from HAECs using an RNeasy Mini kit (Qiagen) following the instructions from the manufacturer. Purification and quantification of RNA were assessed by $A_{260}/A_{280}$ absorption, and an aliquot of RNA (10 μg) from samples with an absorbance ratio above 1.6 was fractionated using a 1.2% agarose formaldehyde gel. Northern blotting of ICAM-1 mRNA was performed as previously described.[28]

### *C pneumoniae* and Mitogen-Activated Protein Kinase Pathway

Cell protein lysates were prepared as described in previous sections. Immunoblotting was performed using PhosphoPlus P44/42, p38, and SAPK/JNK antibody kits (Cell Signaling Technology) and following the manufacturer's instructions.

### *C pneumoniae* and NF-κB Pathway

Nuclear extracts were prepared from infected or controls confluent HAECs ($1.5\times10^6$ cells) as described before.[27,29] Forty micrograms of protein were electrophoresed under reducing conditions on a 10% SDS-polyacrylamide gel. Immunoblotting was performed using an antibody (1 μg/mL) to the activated p65 subunit of NF-κB (Zymed). Immunoblotting of phosphorylated IκB-α was performed using an antibody at 1:100 dilution against human IκB-α that contains the phosphorylated Ser-32 amino acid (Santa Cruz Biotechnology).

### Electrophoretic Mobility Shift Assay

To determine nuclear translocation and binding of NF-κB, electrophoretic mobility shift assay (EMSA) was performed using the Gel Shift Assay Systems (Promega). The oligonucleotide containing the NF-κB consensus sequence probe was labeled with [γ-$^{32}$P]ATP (Perkin Elmer Life Sciences Inc) using T4 polynucleotide kinase (Promega) and was purified by Sephadex G-25 chromatography (Roche Diagnostics Corp). Nuclear extracts (6 μg) from HeLa cells (controls) and noninfected and *C pneumoniae*–infected HAECs, prepared as previously described,[27,29] were incubated at room temperature for 15 minutes with the Gel Shift Binding 5X Buffer provided by the manufacturer before the addition of the radiolabeled NF-κB oligonucleotide probe. The protein-oligonucleotide complexes were then electrophoresed in a 4% nondenaturing polyacrylamide gel, and the radioactive bands were visualized by autoradiography.

### *C pneumoniae* and the Protein Kinase Pathway

Confluent HAECs ($3\times10^6$ cells) were incubated with *C pneumoniae* (1.5 IFU/cell) for 5, 15, 30, and 60 minutes at 37°C, 5% $CO_2$, on a rocker platform. Noninfected cells and cells treated with phorbol, 12-myristate 13 acetate (PMA) (Calbiochem) were used as controls. After incubation, HAECs were washed with PBS/vanadate, and membrane and cytosolic fractions were separated as described.[30] PKC activity was determined by immunoblotting using a purified rabbit polyclonal antibody raised against the PKC-specific peptide (AYQPYGKSVD), a sequence found in the C4 conserved region of PKC (Oncogenes Research Products). PKC isoforms (PKC-α, -β, -δ, -ε, -γ, -η, -ι, -λ, -θ) were detected using a PKC sampler kit (BD Transduction Laboratories).

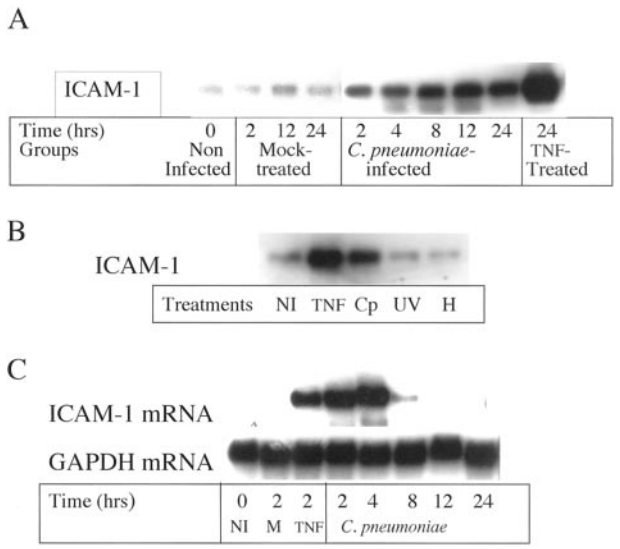


**Figure 1.** Time course expression of ICAM-1 in *C pneumoniae*–infected HAECs. A, Confluent HAECs ($2\times10^5$ cells) were infected with *C pneumoniae* ($8\times10^5$ IFU) for 2 hours and then incubated for the amount of time indicated. B, HAECs were infected with live *C pneumoniae* elementary bodies (Cp) or with either heat- (H) or ultraviolet- (UV) inactivated elementary bodies for 18 hours. Noninfected (NI), mock-treated, and TNF-treated (TNF) HAECs were used as controls. Whole-cell lysates were prepared and electrophoresed on 10% SDS-PAGE, and membranes were immunoblotted with an anti-human ICAM-1 antibody as described in Materials and Methods. C, Northern blot analysis of the time course stimulation of ICAM-1 mRNA in *C pneumoniae*–infected HAECs. HAECs ($1.5\times10^6$ cells) were infected with *C pneumoniae* ($16\times10^6$ IFU) for the amount of time indicated. Noninfected (NI) cells, mock cells (M), and cells treated with TNF (25 U/mL) were used as controls. After completion of the experiments, RNA was isolated as described in Materials and Methods, and 10 $\mu$g RNA was subject to electrophoresis on 1.2% agarose formaldehyde gel and transferred to a nylon membrane. The ICAM-1 and GAPDH mRNAs immobilized on the membrane were hybridized with $^{32}$P-labeled cDNAs as described in Materials and Methods. All panels are representative of 3 separate experiments

An expanded Materials and Methods section can be found in the online data supplement, available at http://www.circresaha.org.

## Results

### Time-Dependent Upregulation of ICAM-1 Expression in *C pneumoniae*–Infected HAECs

As shown in Figure 1A, HAECs infected with *C pneumoniae* showed an increase in ICAM-1 protein expression 4 hours after infection with a peak at 12 hours after infection. High expression of ICAM-1 was still observed up to 24 hours after infection (Figure 1A). Heat and UV inactivation of *C pneumoniae* elementary bodies completely abolished the upregulation of ICAM-1 (Figure 1B).

Figure 1C shows that ICAM-1 mRNA levels were not detectable in either noninfected control cells or in cells exposed to noninfected Hep-2 cells (mock cells). *C pneumoniae*–infected cells have considerably increased ICAM-1 mRNA levels compared with both noninfected and mock cells. The maximum increase in ICAM-1 mRNA levels was observed within 2 to 4 hours after the addition of *C pneumoniae*. The levels declined to near baseline levels after 8 hours of incubation. At 24 hours of incubation, ICAM-1 mRNA was no longer detected (Figure 1C).

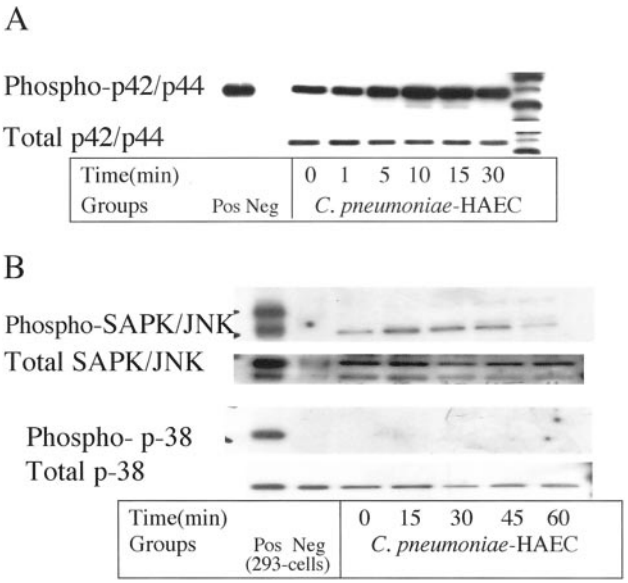


**Figure 2.** Time-dependent activation of the MAPK pathway in *C pneumoniae*–infected HAECs. Confluent HAECs ($2\times10^5$ cells) were stimulated with $6\times10^5$ IFU of *C pneumoniae* for the amount of time indicated. Positive and negative cell extracts obtained from the manufacturer and noninfected (0) HAECs were used as controls. Cell lysates prepared as described in Materials and Methods were electrophoresed on a 12% SDS-PAGE, and the membranes were immunoblotted with antiphosphorylated or anti-total p42/p44 (A), SAPK/JNK, or p38 MAPK (B) antibodies, as described in Materials and Methods. Data are representative of 3 separate experiments with similar results.

### Upregulation of ICAM-1 Expression in *C pneumoniae*–Infected HAECs Is Not Mediated by MAPK Activation

As shown in Figure 2A, increased phosphorylation of extracellular signal regulated kinase (ERK), mainly ERK1, was observed in HAECs infected by *C pneumoniae*. ERK activation was time dependent, peaking at 15 minutes after infection (Figure 2A). A slight activation of SAPK/JNK was also observed at 15 and 30 minutes after infection. Activation of p38 MAPK was not observed at any time point (Figure 2B).

Inhibition of the MAPK pathway by MEK1/2 inhibitors (U0126 and PD98059) did not prevent upregulation of ICAM-1 expression in *C pneumoniae*–infected HAECs (Figures 3A and 3B), although, as expected, ERK phosphorylation was inhibited (Figure 3C). Inhibition of the JNK pathway using SP600125 also failed to inhibit the upregulation of ICAM-1 expression in *C pneumoniae*–infected HAECs (data not shown).

### Inhibition of NF-$\kappa$B Activation Completely Abolishes *C pneumoniae*–Induced ICAM-1 Expression by HAECs

As shown in Figure 4A, in HAECs infected by *C pneumoniae*, nuclear translocation of NF-$\kappa$B occurs in a time-dependent manner, with maximal activation occurring between 30 and 60 minutes after addition of *C pneumoniae* to the

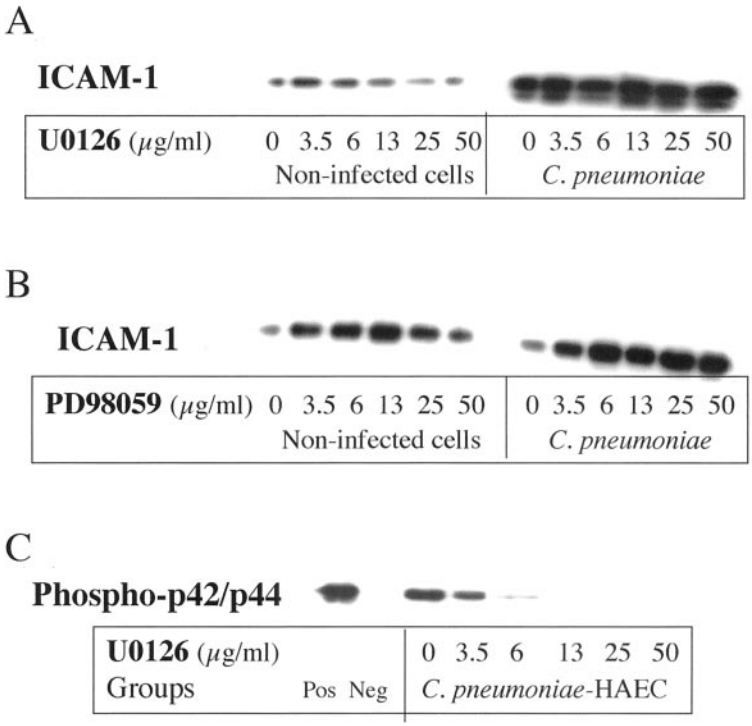


**Figure 3.** Effect of inhibition of the MAPK pathway on ICAM-1 expression by *C pneumoniae*–infected HAECs. HAECs ($2.5\times10^5$ cells) were pretreated with either U0126 (A and C) or PD98059 (B) for 1 hour at concentrations ranging from 0 to 50 μg/mL. Afterward, HAECs were infected with *C pneumoniae* ($6\times10^5$ IFU) for 6 hours (A and B) or 45 minutes (C). Cell lysates were prepared, electrophoresed, and immunoblotted as described in Materials and Methods using an antibody against human ICAM-1 or against phosphorylated p42/p44. Cell lysates provided by the manufacturer were used as positive and negative controls. Data are representative of 2 separate experiments run in duplicate.

HAECs (Figure 4A). We have also shown, as described later in this section, that *C pneumoniae* induces NF-κB activation by inducing phosphorylation of I-κBα (see Figure 6B).

Pretreatment of HAECs for 1 hour with BAY117085 (BAY), a NF-κB inhibitor, reduced the expression of ICAM-1 induced by *C pneumoniae* in a concentration-dependent manner, with complete inhibition at 10 mmol/L concentration (Figure 4B). These findings were confirmed in experiments using another NF-κB inhibitor, CAPE, a specific inhibitor that prevents the translocation of the p65 subunit of NF-κB to the nucleus and therefore inhibits its binding to the DNA. As shown in Figure 4C, activation of ICAM-1 by *C pneumoniae* was completely blocked by CAPE in a concentration-dependent manner. Addition of CAPE at the

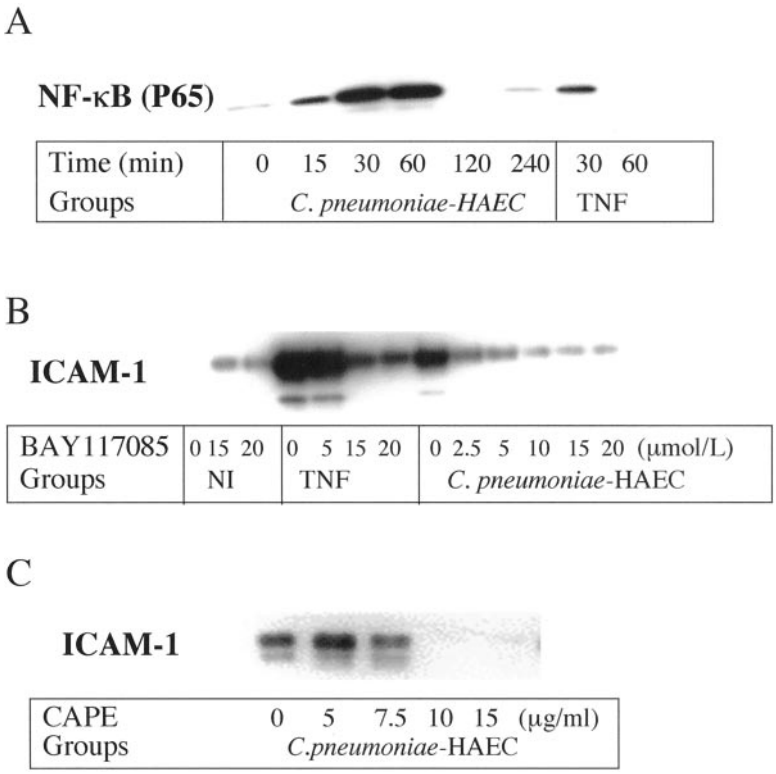


**Figure 4.** Inhibition of NF-κB activation completely abolishes *C pneumoniae*–induced ICAM-1 expression. A, Confluent HAEC monolayers ($1.5\times10^6$ cells) were stimulated with *C pneumoniae* ($16\times10^6$ IFU) and with TNF (100U/mL) for the amount of time indicated. Nuclear proteins were prepared and immunoblotted as described in Materials and Methods using an antibody against the p65 subunit of NF-κB. (B and C). HAECs were pretreated for 1 hour with BAY117085 (B) or CAPE (C) at the concentrations indicated. After incubation, cells were treated for 6 hours with either TNF (25U/mL) or with *C pneumoniae*. Cell lysates were prepared, electrophoresed, and immunoblotted as described in Materials and Methods using an anti-human ICAM-1 antibody. Noninfected cells incubated with similar concentrations of NF-κB inhibitors were used as controls. Data are representative of 2 separate experiments run in duplicate.

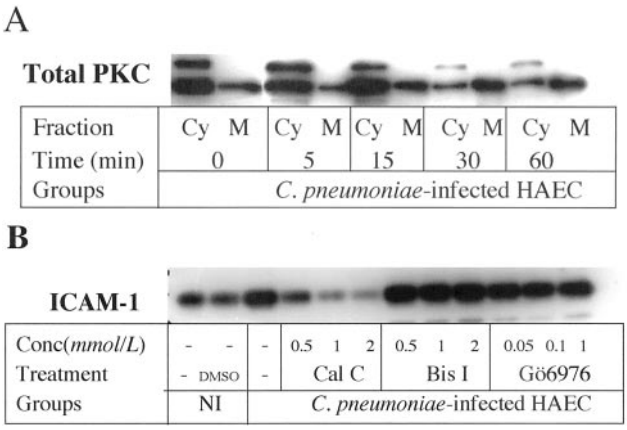


**Figure 5.** *C pneumoniae* activated the PKC pathway and induced ICAM-1 expression via PKC-dependent activation of NF-κB. A, Confluent HAECs ($3\times10^6$ cells) were stimulated with *C pneumoniae* ($32\times10^6$ IFU) for the amount of time indicated. Cytosolic (Cy) and membrane (M) fractions were prepared and used for total PKC detection by Western blot, as described in Materials and Methods. B, Confluent HAEC monolayers ($2\times10^5$ cells) were pretreated for 60 minutes with calphostin C (Cal C), Bisindolylmaleimide I (Bis I), and Gö 6976, followed by infection with *C pneumoniae* ($8\times10^5$ IFU) for 6 hours. Noninfected (NI) and DMSO-treated HAECs were used as controls. Cell lysates were prepared, electrophoresed, and immunoblotted as described in Materials and Methods using an anti-human ICAM-1 antibody. Data are representative of 2 separate experiments run in duplicate.

same concentrations to noninfected HAECs did not affect ICAM-1 protein (data not shown).

### ICAM-1 Upregulation in *C pneumoniae*–Stimulated HAECs Is PKC Dependent

HAECs infected by *C pneumoniae* showed translocation of total PKC from the cytosol to the membrane as early as 30 minutes after addition of *C pneumoniae*. Significant decrease of PKC in the cytosolic fraction and corresponding increase in the membrane fraction was observed both at 30 and 60 minutes after infection (Figure 5A).

Pretreatment of HAECs with calphostin C, which interacts with the common protein's regulatory domain in all isozymes of PKC, prevented *C pneumoniae*–induced ICAM-1 upregulation in a concentration-dependent manner (Figure 5B). At lower concentrations (0.5 mmol/L), calphostin C prevented >50% of the ICAM-1 upregulation induced by *C pneumoniae*, but at higher concentrations (1 and 2 mmol/L), calphostin C not only prevented ICAM-1 upregulation but also decreased ICAM-1 expression below baseline levels (Figure 5B). Pretreatment of HAECs with bisindolylmaleimide I and indolocarbazole Gö 6976, which inhibit the classical and novel PKC isoforms, failed to prevent *C pneumoniae*–induced ICAM-1 expression. Similarly, pretreatment with PMA for 24 hours, which downregulates classical and novel PKC isoforms but not atypical PKCs, did not abolish ICAM-1 expression induced by *C pneumoniae* (data not shown).

### PKC Mediates the Activation of NF-κB in *C pneumoniae*–Infected HAECs

Because both PKC and NF-κB pathways seem to regulate ICAM-1 expression, we examined the effect of PKC on NF-κB activation. Figure 6A shows low levels of NF-κB (p65) in the nuclear extracts from uninfected HAECs. Thirty to sixty minutes after HAECs were infected by *C pneumoniae*, a dramatic increase in NF-κB (p65) was observed. NF-κB (p65) was reduced by more than 80% with either calphostin C (PKC inhibitor) or BAY117085 (NF-κB inhibitor). Experiments using the NF-κB and the PKC inhibitors simultaneously were toxic for HAECs.

Furthermore, as shown in Figure 6B, calphostin C prevents *C pneumoniae*–induced phosphorylation of IκB-α in a concentration-dependent manner. *C pneumoniae* induces nuclear translocation of NF-κB within 15 to 60 minutes (Figures 6A and 6C); in presence of calphostin C (1 and 2 mmol/L), complete inhibition of the *C pneumoniae* translocation of NF-κB to the nucleus was observed (Figure 6C).

## Discussion

Endothelial dysfunction is not only the first step in the development of arteriosclerosis but also contributes to a critical late step, the formation of thrombi that may lead to vessel occlusion and acute cardiovascular events. Thus, it is not surprising that much effort has been focused on determining how infection of the endothelium by *C pneumoniae* causes endothelial dysfunction. Several mechanisms have been described; one of the best studied is the increased expression of ICAM-1. Our present data add to previously published data by demonstrating for the first time that *C pneumoniae* upregulates ICAM-1 expression by PKC-dependent activation of the NF-κB pathway. This study also shows that upregulation of ICAM-1 by *C pneumoniae* requires live bacteria, because heat-killed and UV-inactivated microorganisms did not induce ICAM-1 upregulation in HAECs. The effect of *C pneumoniae* in ICAM-1 upregulation is also specific of this microorganism, because mock-infected HAECs did not show a significant upregulation of ICAM-1 at mRNA or protein level.

We have also demonstrated that although, as previously reported, *C pneumoniae* induces significant activation of the classical MAPK pathway in HAECs,[10] the activation of this pathway is not responsible for the upregulation of ICAM-1 expression. Obviously, the signaling pathways that mediate upregulation of ICAM-1 differ according to cell type. In TNF-stimulated HUVECs, blocking ERK1/2[31] significantly reduced the expression of cell adhesion molecules. However, Chen et al[7,32] found that although TNF and IL-1β led to activation of ERK1/2, as well as upregulation of ICAM-1 expression in alveolar epithelial cells, blocking this pathway did not affect ICAM-1 expression. Similarly, in our studies, the borderline activation by *C pneumoniae* of the SAPK/JNK pathway was not responsible for the upregulation of ICAM-1 in HAECs. However, in Sertori cells, activation of the JNK/SAPK pathway led to upregulation of ICAM-1.[33]

It had been previously shown that NF-κB elements within the proximal ICAM-1 promoter region mediate the increased expression of ICAM-1 in HUVECs and epithelial cells exposed to cytokines (TNF and IL-1β), oxidized LDL, and bacterial lipopolysaccharide.[32,34] We have now shown that *C pneumoniae* leads also to upregulation of ICAM-1 in HAECs through NF-κB activation. Our results show that *C pneu-*

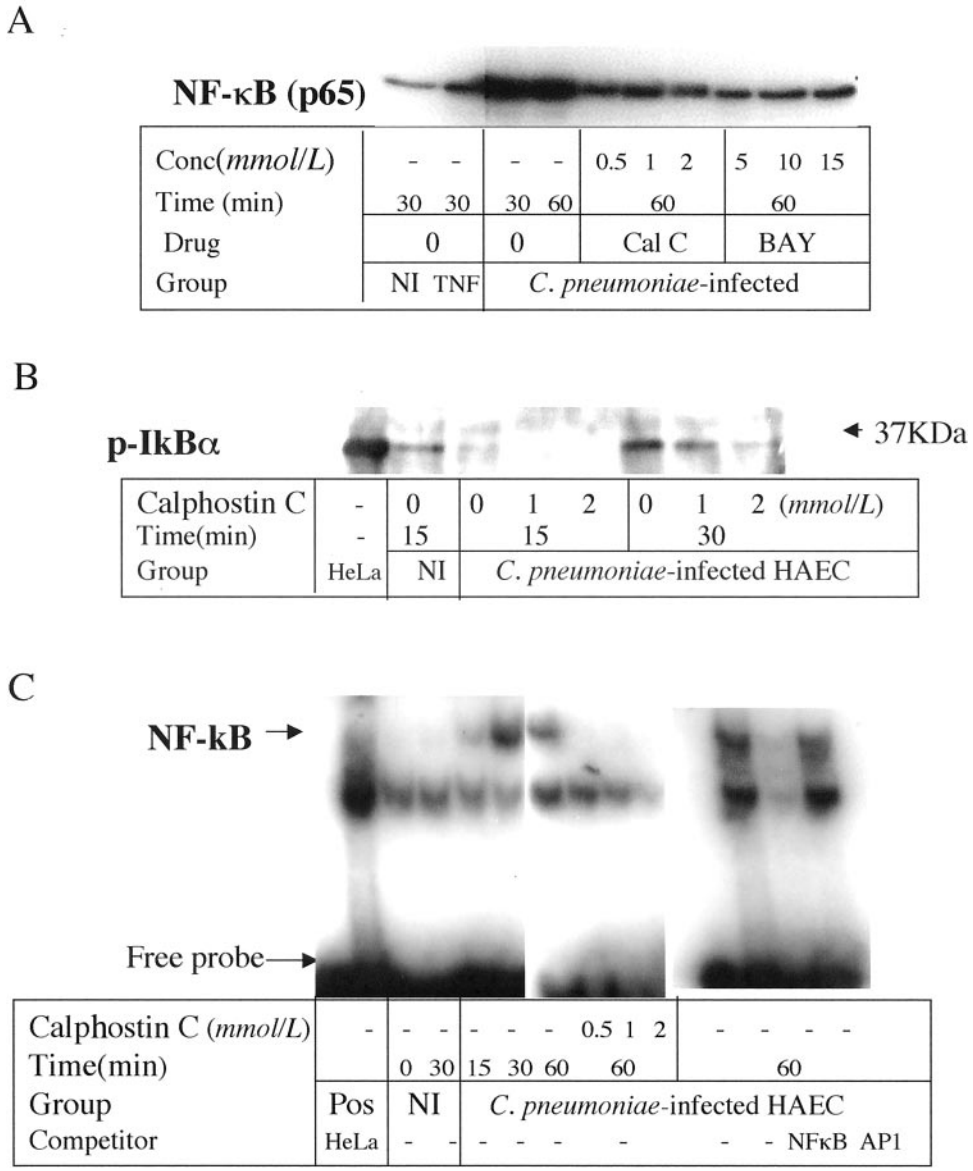


**Figure 6.** *C pneumoniae*–induced ICAM-1 upregulation via PKC-dependent activation of NF-κB. A, Confluent HAECs ($1.5\times10^6$ cells) were pretreated with calphostin C (Cal C) or BAY117085 (BAY) for 60 minutes, followed by stimulation with *C pneumoniae* ($16\times10^6$ IFU) for 60 minutes. Noninfected cells (NI) and TNF-treated (100 U/mL) HAECs for 30 minutes were used as controls. Nuclear protein extracts were prepared, electrophoresed, and immunoblotted as described in Materials and Methods using an antibody against the p65 subunit of NF-κB. B, Confluent HAECs ($2\times10^5$ cells) were pretreated with calphostin C (Cal C), followed by stimulation with *C pneumoniae* for 15 and 45 minutes. Noninfected cells (NI) and TNF-treated (100 U/mL) HAECs were used as controls. Whole-cell protein extracts were analyzed by Western blot using an antibody against the phosphorylated form of IκBα. C, Confluent HAECs ($1.5\times10^6$ cells) were pretreated with calphostin C (Cal C) followed by stimulation with *C pneumoniae*. Nuclear proteins were extracted and analyzed by EMSA for NF-κB. Nuclear extracts from HeLa cells were used as controls. To demonstrate the specificity of binding of the NF-κB oligonucleotide, 50-fold molar excess unlabeled NF-κB or AP-1 were used to compete with the labeled NF-κB probe. Data are representative of 2 experiments.

*moniae* stimulated translocation of NF-κB in HAECs and that ICAM-1 expression was significantly reduced when HAECs were preincubated with specific NF-κB inhibitors such as BAY117085, a novel compound shown to prevent cytokine-induced IκBα phosphorylation[35] and, as a consequence, the cytokine-induced expression of cell adhesion molecules.[35]

Like ICAM-1 and other adhesion molecules (VCAM-1 and E-selectin), many other genes expressed in atheromatous plaques are regulated by NF-κB,[36] including those involved in chemotaxis (monocyte chemotactic protein 1) and thrombogenesis (tissue factor). More recently, translocation of NF-κB after *C pneumoniae* infection was also reported in endothelial cells, smooth muscle cells, and human monocytic cell lines.[23,37,38] Molestina et al[39] demonstrated that in *C pneumoniae*–infected HUVECs, there is increased transcription of the monocyte chemotactic protein 1 via NF-κB activation. Requirement for NF-κB in the induction of tissue

factor and PAI-1 expression by *C pneumoniae* has also been demonstrated in smooth muscle cells.[23]

Finally, we were able to demonstrate for the first time that PKC is activated in HAECs after their infection with *C pneumoniae*. Using 3 PKC inhibitors (calphostin C, bisindolymaleimide I, and indolocarbazole Gö 6976), we investigated the role of PKC in ICAM-1 upregulation. Each of the 3 inhibitors has unique structural characteristics and a unique mechanism of action. Calphostin C interacts with the common protein regulatory domain of all PKC isozymes and therefore inhibits all PKC isoenzymes. Our data showed that calphostin C inhibits ICAM-1 expression in *C pneumoniae*–stimulated HAECs in a concentration-dependent manner, thus indicating that ICAM-1 upregulation by HAECs is PKC dependent. However, neither bisindolymaleimide 1, which selectively inhibits the classical and, at higher concentrations, the novel isoforms of PKC, nor indolocarbazole Gö 6976, a highly selective inhibitor of the classical PKC isoforms, blocked the upregulation of ICAM-1 expression. Although PKC-$\alpha$ and -$\delta$ were activated in HAECs stimulated with *C pneumoniae* (see the online data supplement), our studies with specific inhibitors seem to suggest that neither of these isoforms is involved in the upregulation of ICAM-1. Thus, it is likely that *C pneumoniae* induces ICAM-1 upregulation in HAECs via other novel or atypical PKC isoforms.[27] Rahman et al,[27] using a dominant-negative approach, were able to demonstrate in TNF-stimulated endothelial cells that the atypical isoform PKC$\zeta$ was able to prevent I$\kappa$B$\alpha$ degradation and NF-$\kappa$B binding to the ICAM-1 promoter.[27] We were unable to confirm the involvement of other PKC isoforms because of the unavailability of specific inhibitors.

However, based on the above data and on some experimental evidence suggesting that PKC activation leads to the dissociation of the NF-$\kappa$B/IkB complex and therefore to the translocation of NF-$\kappa$B to the nucleus and activation of its target genes,[40] we investigated the possible effect of PKC activation by *C pneumoniae* on the NF-$\kappa$B inhibitor, I-$\kappa$B$\alpha$. Inhibition of the PKC pathway in *C pneumoniae*–infected HAECs by calphostin C blocked ICAM-1 upregulation by preventing I-$\kappa$B$\alpha$ phosphorylation and, as a consequence, the translocation of NF-$\kappa$B to the nucleus. The degree of inhibition of NF-$\kappa$B by calphostin C was similar to that induced by the specific NF-$\kappa$B inhibitor BAY117085. A similar pathway regulates the events that modulate adhesion and filtration of leukocytes and upregulation of ICAM-1 expression on HUVECs and mesangial cells exposed to high levels of glucose.[37,41] Hence, in *C pneumoniae*–infected HAECs, we demonstrate that PKC enhances NF-$\kappa$B activity by direct phosphorylation of its cytoplasmic inhibitor, I-$\kappa$B$\alpha$, thus facilitating NF-$\kappa$B binding to the NF-$\kappa$B motif in the ICAM-1 promoter.

In conclusion, the present study shows for the first time that PKC is activated in HAECs on infection with *C pneumoniae*. Activation of PKC leads to NF-$\kappa$B activation, and that, in turn, leads to increased transcription of the ICAM-1 gene. Experiments designed to additionally elucidate the mechanism of selective induction of ICAM-1 signaling pathways by *C pneumoniae* and the role of different cell lines in this process are presently ongoing in our laboratory. *C pneumoniae*–induced activation of ICAM-1 is likely to contribute to the chronic inflammatory events associated with arteriosclerosis.

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## References

1. Casasnovas JM, Stehle T, Liu JH, Wang JH, Springer TA. A dimeric crystal structure for the N-terminal two domains of intercellular adhesion molecule-1. *Proc Nat Acad Sci U S A*. 1998;95:4134–4139.
2. Kevil CG, Patel RP, Bullard DC. Essential role of ICAM-1 in mediating monocyte adhesion to aortic endothelial cells. *Am J Physiol Cell Physiol*. 2001;281:C1442–C1447.
3. Hubbard AK, Rothlein R. Intercellular adhesion molecule-1 (ICAM-1) expression and cell signaling cascades. *Free Radic Biol Med*. 2000;28: 1379–1386.
4. Roebuck KA, Finnegan A. Regulation of intercellular adhesion molecule-1 (CD54) gene expression. *J Leukoc Biol*. 1999;66:876–888.
5. Lakshminarayanan V, Beno DWA, Costa RH, Roebuck KA. Differential regulation of interleukin-8 and intercellular adhesion molecule-1 by $H_2O_2$ and tumor necrosis factor-$\alpha$ in endothelial and epithelial cells. *J Biol Chem*. 1997;272:32910–32918.
6. Khan BV, Parthasarathy SS, Alexander RW, Medford RM. Modified low density lipoprotein and its constituents augment cytokine-activated vascular cell adhesion molecule-1 gene expression in human vascular endothelial cells. *J Clin Invest*. 1995;95:1262–1270.
7. Chen C-C, Chou C-Y, Sun Y-T, Huang W-C. Tumor necrosis factor $\alpha$-induced activation of downstream NF-$\kappa$B site of the promoter mediates epithelial ICAM-1 expression and monocyte adhesion: involvement of PKC-$\alpha$, tyrosine kinase, and IKK2 but not MAPKs, pathway. *Cell Signal*. 2001;13:543–553.
8. Takei A, Huang Y, Lopes-Virella MF. Expression of adhesion molecules by human endothelial cells exposed to oxidized low density lipoprotein: influences of degree of oxidation and location of oxidized LDL. *Atherosclerosis*. 2001;154:79–86.
9. Papi A, Johnston SL. Rhinovirus infection induces expression of its own receptor intercellular adhesion molecule 1 (ICAM-1) via increased NF-$\kappa$B-mediated transcription. *J Biol Chem*. 1999;274:9707–9720.
10. Krüll M, Klucken AC, Wuppermann FN, Fuhrmann O, Magerl C, Seybold J, Hippenstiel S, Hegemann JH, Jantos CA, Suttorp N. Signal transduction pathways activated in endothelial cells following infection with *Chlamydia pneumoniae*. *J Immunol*. 1999;162:4834–4841.
11. Chia MC. The role of adhesion molecules in atherosclerosis. *Crit Rev Clin Lab Sci*. 1998;35:573–602.
12. Saikku O, Mattila K, Nieminen MS, Makela PH, Huttunen JK, Valtonen V. Serological evidence of an association of a novel chlamydia TWAR with chronic coronary heart disease and acute myocardial infarction. *Lancet*. 1988;2:983–986.
13. Shor A, Kuo CC, Patton DL. Detection of *Chlamydia pneumoniae* in coronary arterial fatty streaks and atheromatous plaques. *S Afr Med J*. 1992;82:158–161.
14. Muhlestein JB. Chronic infection and coronary artery disease. *Med Clin North Am*. 2000;84:123–148.
15. Campbell LA, Kuo CC, Grayston JT. *Chlamydia pneumoniae* and cardiovascular disease. *Emerg Infect Dis*. 1998;4:571–579.
16. Gaydos CA, Summersgill JT, Sahney NN, Ramirez J, Quinn TC. Replication of *Chlamydia pneumoniae* in vitro in human macrophages, endothelial cells, and aortic artery smooth muscle cells. *Infect Immunol*. 1996;64:1614–1620.
17. Godzik KL, O'Brien ER, Wang SK, Kuo CC. In vitro susceptibility of human vascular wall cells to infection with *Chlamydia pneumoniae*. *J Clin Microbiol*. 1995;33:2411–2414.
18. Bodetti TJ, Timms P. Detection of *Chlamydia pneumoniae* DNA and antigen in the circulating mononuclear cell fractions of human and koalas. *Infect Immunol*. 2000;68:2744–2747.

19. Gaydos CA. Growth in vascular cells and cytokine production by *Chlamydia pneumoniae*. *J Infect Dis*. 2000;181:S473–S478.
20. Kaukoranta-Tolvanen SSE, Ronni T, Leinonen M, Saikku P, Laitinen K. Expression of adhesion molecules on endothelial cells stimulated by *Chlamydia pneumoniae*. *Microb Pathog*. 1996;21:407–411.
21. Molestina RE, Dean D, Miller RD, Ramirez JA, Summersgill JT. Characterization of a strain of *Chlamydia pneumoniae* isolated from coronary atheroma by analysis of the omp1 gene and biological activity in human endothelial cells. *Infect Immunol*. 1998;66:1370–1376.
22. Molestina RE, Miller RD, Ramirez JA, Summersgill JT. Infection of human endothelial cells with *Chlamydia pneumoniae* stimulates transendothelial migration of neutrophils and monocytes. *Infect Immunol*. 1999; 67:1323–1330.
23. Dechend R, Maass M, Gieffers J, Dietz R, Scheidereit C, Leutz A, Gulba DC. *Chlamydia pneumoniae* infection of vascular smooth muscle and endothelial cells activates NF-κB and induces tissue factor and PAI-I expression. *Circulation*. 1999;100:1369–1373.
24. Vielma S, Gorod AJ, Virella G, Lopes-Virella MF. *Chlamydophila pneumoniae* infection of human aortic endothelial cells induces the expression of Fc-γ receptor II (FcγRII). *Clin Immunol*. 2002;104:265–273.
25. Kuo CC, Grayston JT. A sensitive cell line, HL cells, for isolation and propagation of *Chlamydia pneumoniae* strain TWAR. *J Infect Dis*. 1990; 162:755–758.
26. Wong KH, Skelton S, Chan YK. Efficient culture of *Chlamydia pneumoniae* with cell lines derived from the human respiratory tract. *J Clin Microbiol*. 1992;30:1625–1630.
27. Rahman A, Anwar KN, Malik AB. Protein kinase C-ζ mediates TNF-α-induced ICAM-1 gene transcription in endothelial cells. *Am J Physiol Cell Physiol*. 2000;279:C906–C914.
28. Huang Y, Mironova M, Lopes-Virella MF. Oxidized LDL stimulates matrix metalloproteinase-1 expression in human vascular endothelial cells. *Arterioscler Thromb Vasc Biol*. 1999;19:2640–2647.
29. Martel-Pelletier J, Mineau F, Jovanovic D, DiBattista JA, Pelletier JP. Mitogen-activated protein kinase and nuclear factor κB together regulate interleukin-17-induced nitric oxide production in human osteoarthritic chondrocytes: possible role of transactivating factor mitogen-activated protein kinase-activated protein kinase (MAPKAPK). *Arthritis Rheum*. 1999;42:2399–2409.
30. Rahman A, Bando M, Kefer J, Anwar KN, Malik AB. Protein kinase C-activated oxidant generation in endothelial cells signals intercellular adhesion molecule-1 gene transcription. *Mol Pharmacol*. 1999;55: 575–583.
31. Xia P, Gamble JR, Rye KA, Wang L, Hii CST, Cockerill P, Khew-Goodall Y, Bert AG, Barter PJ, Vadas MA. Tumor necrosis factor-α induces adhesion molecule expression through the sphingosine kinase pathway. *Proc Nat Acad Sci U S A*. 1998;95:14196–14201.
32. Chen C-C, Chen J-J, Chou C-Y. Protein kinase C-α but not p44/p42 mitogen-activated protein kinase, p38, or c-Jun $NH_2$-terminal kinase is required for intercellular adhesion molecules-1 expression mediated by interleukin-1β: involvement of sequential activation of tyrosine kinase, nuclear factor-κB-inducing kinase, and I κB kinase 2. *Mol Pharmacol*. 2000;58:1479–1489.
33. De Cesaris P, Starace D, Starace G, Filippini A, Stefanini M, Ziparo E. Activation of Jun N-terminal kinase/stress-activated protein kinase pathway by tumor necrosis factor α leads to intercellular adhesion molecule-1 expression. *J Biol Chem*. 1999;274:28978–28982.
34. Cominacini L, Pasini AF, Garbin U, Davoli A, Tosetti ML, Campagnola M, Rigoni A, Pastorino AM, Lo Cascio V, Sawamura T. Oxidized low density lipoprotein (ox-LDL) binding to ox-LDL receptor-1 in endothelial cells induces the activation of NF-κB through an increased production of intracellular reactive oxygen species. *J Biol Chem*. 2000;275: 12633–12638.
35. Pierce JW, Schoenleber R, Jesmok G, Best J, Moore SA, Collins T, Gerritsen ME. Novel inhibitors of cytokine-induced I κB-α phosphorylation and endothelial cell adhesion molecule expression show anti-inflammatory effects in vivo. *J Biol Chem*. 1997;272:21096–21103.
36. Denk A, Goebeler M, Schmid S, Berberich I, Ritz O, Lindemann D, Ludwig S, Wirth T. Activation of NF-κB via the IkB kinase complex is both essential and sufficient for proinflammatory gene expression in primary endothelial cells. *J Biol Chem*. 2001;276:28451–28458.
37. Morigi M, Angioletti S, Imberti B. Leukocyte-endothelial interaction is augmented by high glucose concentrations and hyperglycemia in a NF-κB–dependent fashion. *J Clin Invest*. 1998;101:1905–1915.
38. Wahl C, Oswald F, Simnacher U, Weiss S, Marre R, Essig A. Survival of *Chlamydia pneumoniae*–infected Mono Mac 6 cells is dependent on NF-κB binding activity. *Infect Immunol*. 2001;69:7039–7045.
39. Molestina RE, Miller RD, Lentsch AB, Ramirez JA, Summersgill JT. Requirement for NF-κB in transcriptional activation of monocyte chemotactic protein 1 by *Chlamydia pneumoniae* in human endothelial cells. *Infect Immunol*. 2000;68:4282–4288.
40. Ghosh S, Gifford AM, Riviere LR, Tempst P, Nolan GP, Baltimore D. Cloning of the p50 DNA binding subunit of NF-κB: homology to rel and dorsal. *Cell*. 1990;62:1019–1029.
41. Park CW, Kim JH, Lee H, Kim YS, Ahn HJ, Shin YS, Kim SY, Choi EJ, Chang YS, Bang BK, et al. High glucose-induced intercellular adhesion molecules-1 (ICAM-1) expression through an osmotic effect in rat mesangial cells is PKC-NF-κB–dependent. *Diabetologia*. 2000;43:1544–1553.