Chlamydial and human heat shock protein 60s activate human vascular endothelium, smooth muscle cells, and macrophages

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Both chlamydial and human heat shock protein 60s (HSP 60), which colocalize in human atheroma, may contribute to inflammation during atherogenesis. We tested the hypothesis that chlamydial or human HSP 60 activates human endothelial cells (ECs), smooth muscle cells (SMCs), and monocyte-derived macrophages. We examined the expression of adhesion molecules such as endothelial-leukocyte adhesion molecule-1 (E-selectin), intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1), and the production of the proinflammatory cytokine interleukin-6 (IL-6). We also tested whether either HSP 60 induces nuclear factor-κB (NF-κB), which contributes to the gene expression of these molecules. Either chlamydial or human HSP 60 induced E-selectin, ICAM-1, and VCAM-1 expression on ECs similar to levels induced by *Escherichia coli* lipopolysaccharide (LPS). Each HSP 60 also significantly induced IL-6 production by ECs, SMCs, and macrophages to an extent similar to that induced by *E. coli* LPS, as assessed by enzyme-linked immunosorbent assay (ELISA). In ECs, either HSP 60 triggered activation of NF-κB complexes containing p65 and p50 Rel proteins. Heat treatment abolished all these effects, but did not alter the ability of *E. coli* LPS to induce these functions. Chlamydial and human HSP 60s therefore activate human vascular cell functions relevant to atherogenesis and lesional complications. These findings help to elucidate the mechanisms by which a chronic asymptomatic chlamydial infection might contribute to the pathophysiology of atheroma.

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Introduction

The past few years have witnessed increasing interest in the possibility that chronic asymptomatic infection with the airborne agent *Chlamydia pneumoniae* might contribute to atherogenesis and, possibly, to plaque instability and acute coronary syndromes (1–4). *C. pneumoniae* exist within coronary plaques (5, 6). Also, some recent preliminary studies show that treatment with macrolide antibiotics might reduce recurrent coronary events in patients who have sustained acute myocardial infarction (7, 8), and may limit development of atherosclerosis in rabbits (9).

However, the molecular mechanisms by which *C. pneumoniae* might contribute to atheroma formation and evolution remain unclear. We have recently demonstrated that chlamydial heat shock protein 60 (HSP 60), produced in large amounts during chronic chlamydial infections, colocalizes within plaque macrophages with human HSP 60 (10). In addition, we have made the surprising observation that either chlamydial or human HSP 60, normally intracellular proteins with protective function, can act as extracellular agonists and induce tumor necrosis factor-α (TNF-α) and matrix metalloproteinase (MMP-9) production by mouse macrophages (10).

C. pneumoniae can infect human endothelial cells (ECs) (11), where it induces the expression of adhesion molecules like endothelial-leukocyte adhesion molecule-1 (E-selectin), intercellular adhesion molecule-1 (ICAM-1),

and vascular cell adhesion molecule-1 (VCAM-1) (12). *C. pneumoniae* also infects human monocyte–derived macrophages, stimulating the production of proinflammatory cytokines such as TNF-α and interleukin-6 (IL-6) (13). In addition, *C. pneumoniae* can infect human smooth muscle cells (SMCs) (14), although the effects of infection on this cell type have not been studied. In the view of the high levels of expression of chlamydial HSP 60 during chronic, persistent chlamydial infections (15, 16), our recent findings raise the intriguing possibility that chlamydial HSP 60, may activate human vascular cells (ECs, SMCs) and infiltrating macrophages, and may thereby mediate the inflammation that appears to promote atheroma formation and evolution. The induction of adhesion molecules on infected endothelium might aid trafficking of monocytes and lymphocytes in atheroma (17, 18). The induction of proinflammatory cytokines such as TNF-α or IL-6 might promote plaque instability and thrombosis (19, 20). Because human atheroma contain both human and chlamydial HSP 60s (10, 21), and HSP 60s from different species share a substantial sequence similarity (22), chlamydial and human HSP 60s might have similar functions on human vascular cells.

This study therefore tested the hypothesis that chlamydial HSP 60 may activate human vascular cells and macrophages and that human HSP 60 shares similar actions. We examined the expression of adhesion molecules
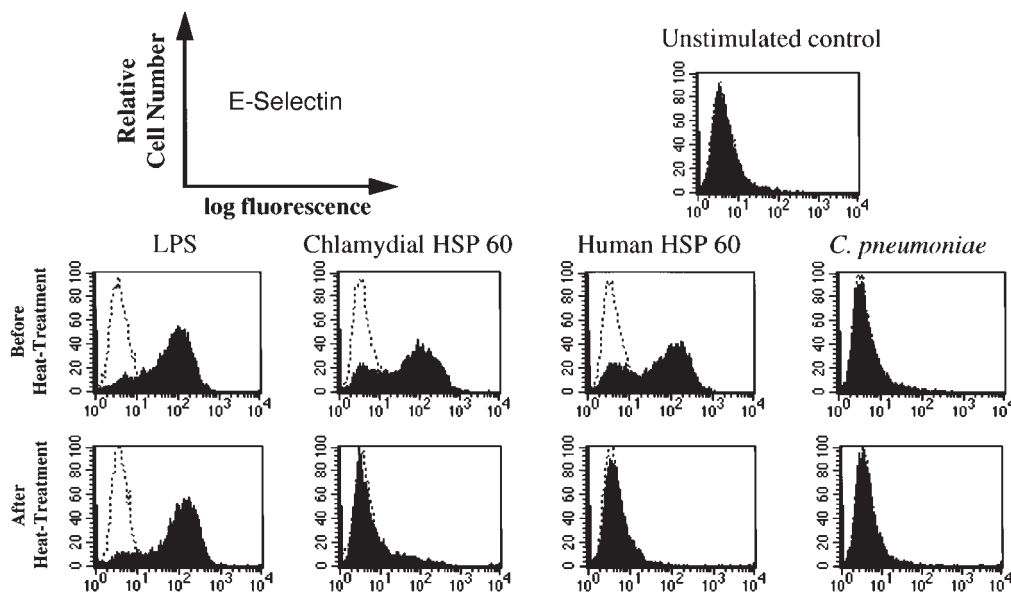
Figure 1

Chlamydial and human HSP 60s induce E-selectin production by endothelial cells. (***top***) ECs were incubated with medium only (unstimulated control), or with chlamydial HSP 60 (5 μg/ml), human HSP 60 (5 μg/ml), *E. coli* LPS (1 μg/ml), or inactivated *C. pneumoniae* (10^7 U/ml), for 6 h. Cells were harvested by treatment with trypsin-EDTA and then stained with antibody against E-selectin (*solid histograms*) or with mouse IgGs (as isotype control; *open histograms*). Chlamydial HSP 60 and human HSP 60 had a similar effect on E-selectin production as *E. coli* LPS. Inactivated *C. pneumoniae* did not elicit E-selectin expression by ECs. (***bottom***) Before incubation, reagents were heat-treated by boiling for 20 min. Heat treatment abolished the effect on E-selectin by chlamydial HSP 60 and human HSP 60, but did not modify the effect of thermostable *E. coli* LPS. Three independent experiments showed similar results. *EC*, endothelial cells; *HSP*, heat shock protein; *LPS*, lipopolysaccharide.

on ECs (E-selectin, ICAM-1, and VCAM-1) and on SMCs (ICAM-1 and VCAM-1) and the production of a proinflammatory cytokine (IL-6) by ECs, SMCs, and monocyte-derived macrophages. Because E-selectin, ICAM-1, VCAM-1, and IL-6 gene expression involves activation of nuclear factor-κB (NF-κB) (23), we also tested the hypothesis that HSP 60s trigger NF-κB activation.

Methods

Reagents. The recombinant *Chlamydia trachomatis* HSP 60 fusion protein expression plasmid was the gift of R. Stephens (University of California–San Francisco, San Francisco, California, USA) (24). Production of chlamydial HSP 60, purification, and site-specific proteolysis with thrombin were performed as described elsewhere (25) using a RediPack GST Purification

Table 1

Percentages of endothelial cells positively stained for E-selectin, ICAM-1, and VCAM-1

	E-selectin	ICAM-1	VCAM-1
Unstimulated control	3%	16%	2%
LPS	88%	93%	29%
LPS (heat-treated)	89%	88%	32%
Chlamydial HSP 60	76%	73%	15%
Chlamydial HSP 60 (heat-treated)	16%	18%	2%
Human HSP 60	74%	71%	15%
Human HSP 60 (heat-treated)	9%	18%	2%
C. pneumoniae	9%	19%	2%
C. pneumoniae (heat-treated)	6%	15%	3%

ICAM-1, intercellular adhesion molecule-1; *VCAM-1*, vascular cell adhesion molecule-1; *LPS*, lipopolysaccharide; *HSP*, heat shock protein.

Module (Pharmacia Biotech, Piscataway, New Jersey, USA). Recombinant human HSP 60 was purchased from StressGen Biotechnologies Corp. (Victoria, British Columbia, Canada). *E. coli* lipopolysaccharide (LPS) was purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). Formalin-inactivated *C. pneumoniae* elementary bodies were obtained from Washington Research Foundation (Seattle, Washington, USA).

Cell isolation and culture. Human vascular ECs from human umbilical cord and SMCs from saphenous veins were isolated and cultured as described (26, 27). Vascular SMCs and ECs were cultured in DMEM and M199 medium (BioWhittaker, Walkersville, Maryland, USA), respectively. Both culture media contained 10% FCS (HyClone Laboratories, Logan, Utah, USA); endothelial cell growth supplement and heparin were added to ECs culture medium (28). Vascular SMCs were cultured 48 h before the experiment in insulin/transferrin medium lacking fetal calf serum (29).

Monocytes were isolated from freshly prepared human peripheral blood mononuclear cells obtained from leukopacs of healthy donors (provided by S.K. Clinton, Ohio State University, Columbus, Ohio, USA) by density gradient centrifugation (30), and by adherence to plastic culture flasks (2 h at 37°C). Monocytes were harvested by incubation with PBS-EDTA for 10 min at 37°C and plated at a density of 5×10^5 cells/cm^2 in RPMI-1640 medium (BioWhittaker) containing 5% human serum (Sigma Chemical Co.). Cells were cultured for 7–10 days, serum was added every 2 days, and medium was changed every 4 days. For specific experiments, macrophages were precultured 18 h in RPMI-1640 lacking serum.

Experimental conditions and preparation of conditioned medium. Cells were incubated with recombinant chlamydial HSP 60, human HSP 60, inactivated *C. pneumoniae*, *E. coli* LPS, or with medium alone as a negative control. In the same experiments, before incubation, a portion of each reagent was heat-treated by boiling for 20 min. Concentrations used and incubation periods are indicated in individual experiments. Concentrations of inactivated *C.*

pneumoniae are expressed as U/ml, which correspond to the number of microorganisms per ml of culture medium. Conditioned medium was collected at different time points and frozen for further analysis. Each experiment was repeated at least three times.

Flow cytometry. Human SMCs and ECs were first harvested by incubation with HBSS containing trypsin-EDTA (Sigma Chemical Co.). Cells were then incubated (30 min at 4°C) with the specific primary antibody: antibodies against human E-selectin (H18/7) (31), ICAM-1 (Hu5/3) (32), and VCAM-1(E1/6) (33) were a generous gift of M. Gimbrone (Brigham and Women's Hospital, Boston, Massachusetts, USA); control mouse IgGs were purchased from PharMingen (San Diego, California, USA). Cells were thereafter incubated (30 min at 4°C) with FITC-conjugated secondary antibodies against mouse IgG$_1$ (Southern Biotechnology Associates, Birmingham, Alabama, USA) and fixed in 1% paraformaldehyde. FACS analysis was performed with a FACScan flow cytometer, and data were analyzed using CELLQUEST software (Becton Dickinson and Co., Franklin Lanes, New Jersey, USA). Data are represented as percentages of positively stained cells.

Cytokine assay. IL-6 levels in culture supernatants were measured by ELISA using paired antibodies purchased from Endogen Inc. (Woburn, Massachusetts, USA). Samples were assayed in triplicate. Antibody binding was detected by adding *P*-nitrophenyl phosphate (1 mg/ml; Sigma Chemical Co.). Absorbance was measured in a Dynatech plate reader at 410 λ. The amount of cytokine detected was calculated from a standard curve prepared from the recombinant cytokine purchased from Endogen Inc.

Data are expressed as mean ± SEM. Differences between experimental conditions were assessed by ANOVA with Bonferroni correction. $P \leq 0.05$ was considered significant.

Isolation of nuclear proteins and electromobility shift assay. Human umbilical vein endothelial cells (HUVECs) grown to confluency in 10-cm^2 dishes were treated with chlamydial or human HSP 60 (2 μg/ml) or with LPS (1 μg/ml) for the indicated time periods. Nuclear proteins were extracted by the method of Dignam (34) with modifications (35). Double-stranded oligonucleotides

that span the NF-κB–binding site of the human IL-6 promoter (GGGATTTTCCC), purchased from Integrated DNA Technologies (Coralville, Iowa, USA), were annealed and labeled with [^{32}P]ATP using T4 polynucleotide kinase. For electromobility shift assay (EMSA), 5 μg of nuclear extract was combined in 20 μl buffer containing 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 5% glycerol, 1 μg poly (dI/dC), and 30,000-cpm labeled probe (0.1–0.5 ng). The mixtures were incubated 30 min at room temperature. For competition reactions, 50 ng of unlabeled probe, consensus or mutant NF-κB oligomer (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA) were added 15 min before addition of labeled probe. Supershifts were performed by adding 1 μg of polyclonal rabbit anti-Rel antibodies or isotype-matched nonimmune IgG (Santa Cruz Biotechnology Inc.) to reactions 20 min before addition of labeled probe. Protein–DNA complexes were resolved on 5% polyacrylamide gels run in 0.5× Tris-borate buffer at 200 V for 2 h. Gels were dried and exposed to autoradiography film for 16–24 h.

Results

Chlamydial and human HSP 60s induce E-selectin, ICAM-1, and VCAM-1 expression on endothelial cells. Expression of adhesion molecules on endothelium and/or smooth muscle cells regulates the leukocyte trafficking within the vascular wall, likely contributing to atherogenesis (17, 18). We therefore tested whether chlamydial or human HSP 60 can induce the expression of E-selectin on ECs, and of ICAM-1 and VCAM-1 on ECs and SMCs. After six hours, either chlamydial or human HSP 60 induced E-selectin on ECs to an extent similar to that induced by *E. coli* LPS; nonstimulated cells expressed very low levels of E-selectin (Fig. 1, *top*; Table 1). After 24 hours, either chlamydial or human HSP 60 induced ICAM-1 on ECs to an extent similar to that induced by *E. coli* LPS; nonstimulated cells expressed low levels of ICAM-1 (Fig. 2, *top*; Table 1).
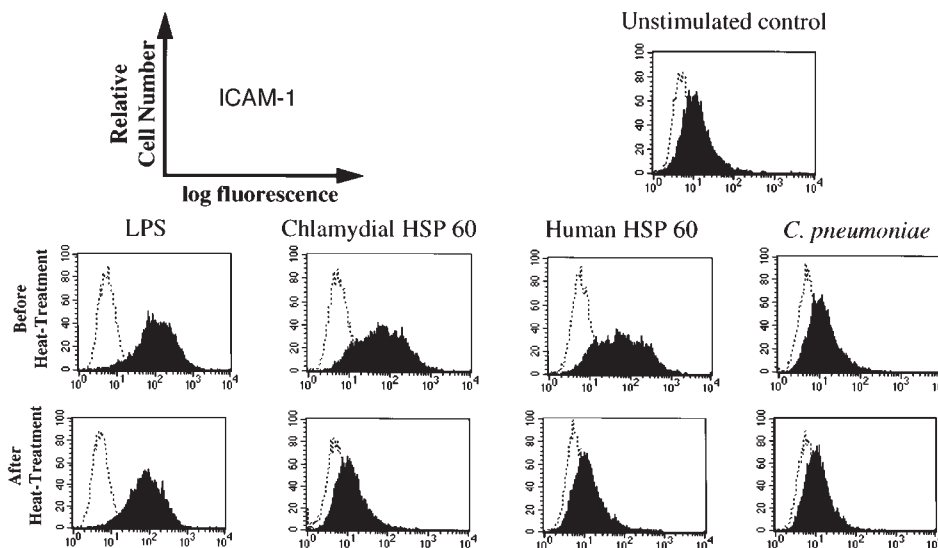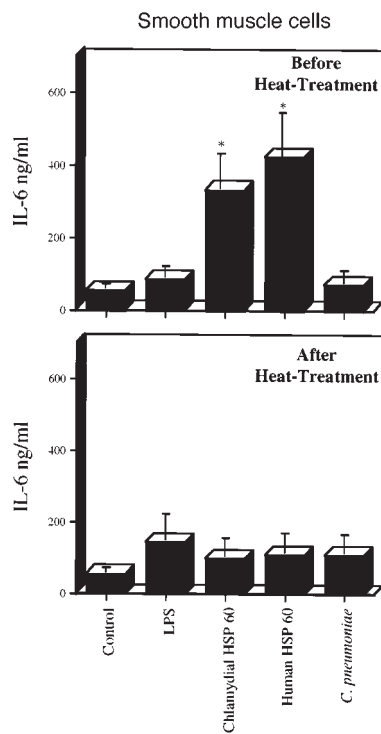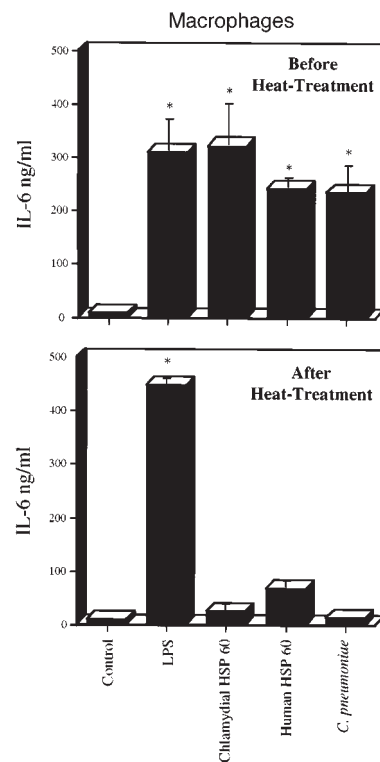

Figure 2

Chlamydial and human HSP 60s induce *ICAM-1* production by endothelial cells. (*top*) ECs were incubated with medium only (unstimulated control), or with chlamydial HSP 60 (5 μg/ml), human HSP 60 (5 μg/ml), *E. coli* LPS (1 μg/ml), or inactivated *C. pneumoniae* (10^7 μg/ml), for 24 h. Cells were harvested by treatment with trypsin-EDTA and stained with antibody against ICAM-1 (*solid histograms*) or with mouse IgGs (as isotype control; *open histograms*). Chlamydial HSP 60 and human HSP 60 had a similar effect on ICAM-1 production as *E. coli* LPS. Inactivated *C. pneumoniae* did not elicit ICAM-1 expression by ECs. (*bottom*) Before incubation, reagents were heat-treated by boiling for 20 min. Heat treatment abolished the effect on ICAM-1 by chlamydial HSP 60 and human HSP 60, but did not modify the effect of thermostable *E. coli* LPS. Three independent experiments showed similar results. *ICAM-1*, intercellular adhesion molecule-1.
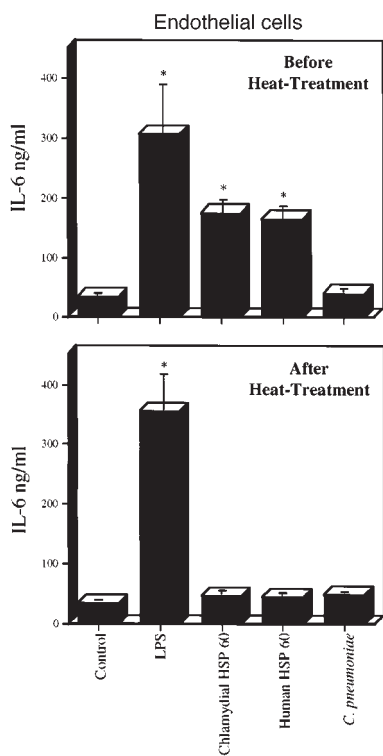
Figure 3
Chlamydial and human HSP 60s induce IL-6 production by endothelial cells. (*top*) ECs were incubated with medium only (unstimulated control), or with chlamydial HSP 60 (5 μg/ml), human HSP 60 (5 μg/ml), *E. coli* LPS (1 μg/ml), or inactivated *C. pneumoniae* (10^7 U/ml), for 24 h. Samples were collected and analyzed for IL-6 by ELISA. Chlamydial HSP 60 and human HSP 60, as well as *E. coli* LPS, significantly increased IL-6 production over control levels. Inactivated *C. pneumoniae* did not elicit IL-6 expression by ECs. (*bottom*) Before incubation, reagents were heat-treated by boiling for 20 min. Heat treatment abolished the effect on IL-6 by chlamydial HSP 60 and human HSP 60, but did not modify the effect of thermostable *E. coli* LPS. Results shown represent the mean ± SEM of three independent experiments. Statistically significant vs. control ($P ≤ 0.01$, two-sided). *IL-6*, interleukin-6.

Figure 4
Chlamydial and human HSP 60s induce IL-6 production by smooth muscle cells. (*top*) SMCs were incubated without serum for 48 h and then with medium only (unstimulated control), or with chlamydial HSP 60 (5 μg/ml), human HSP 60 (5 μg/ml), *E. coli* LPS (1 μg/ml), or inactivated *C. pneumoniae* (10^7 U/ml), for 24 h. Samples were collected and analyzed for IL-6 by ELISA. Chlamydial HSP 60 and human HSP 60 significantly increased IL-6 production over control levels. Neither *E. coli* LPS nor inactivated *C. pneumoniae* did elicit IL-6 expression by SMCs. (*bottom*) Before incubation, reagents were heat-treated by boiling for 20 min. Heat treatment abolished the effect on IL-6 by both chlamydial HSP 60 and human HSP 60. Results shown represent the mean ± SEM of three independent experiments. Statistically significant vs. control ($P ≤ 0.05$, two-sided). *SMCs*, smooth muscle cells.

Figure 5
Chlamydial and human HSP 60s induce IL-6 production by monocyte-derived macrophages. (*top*) Macrophages were incubated with medium only (unstimulated control), or with chlamydial HSP 60 (5 μg/ml), human HSP 60 (5 μg/ml), *E. coli* LPS (1 μg/ml), or inactivated *C. pneumoniae* (10^7 U/ml), for 24 h. Samples were collected and analyzed for IL-6 by ELISA. Chlamydial HSP 60 and human HSP 60, as well as *E. coli* LPS and inactivated *C. pneumoniae*, significantly increased IL-6 production over control levels. (*bottom*) Before incubation, reagents were heat-treated by boiling for 20 min. Heat treatment abolished the effect on IL-6 by chlamydial HSP 60, human HSP 60, and inactivated *C. pneumoniae*, but did not modify the effect of thermostable *E. coli* LPS. Results shown represent the mean ± SEM of three independent experiments. Statistically significant vs. control ($P ≤ 0.01$, two-sided).

Chlamydial or human HSP 60 also induced VCAM-1 on ECs, as did *E. coli* LPS, although the level of expression was lower than for E-selectin and ICAM-1 (Table 1). In contrast, SMCs showed no increase over basal levels of either ICAM-1 or VCAM-1 (data not shown).

Infection of ECs with live *C. pneumoniae* induces the expression of E-selectin, ICAM-1, and VCAM-1 (12). However, it is not clear whether this effect is mainly due to membrane constituents, such as LPS or the major outer membrane protein (MOMP), or to cytoplasmic constituents, such as the chlamydial HSP 60. Under the conditions used in this study, cells exposed to formalin-inactivated *C. pneumoniae*, which retain antigenicity (10) but cannot enter host cells, did not

show increased expression of E-selectin, ICAM-1, or VCAM-1 (Figs. 1 and 2, *top*; Table 1). This observation suggests that a cytoplasmic component, such as the chlamydial HSP 60, and not a membrane constituent, may be responsible for the observed effects on ECs after infection with live *C. pneumoniae*.

Both the chlamydial and human HSP 60s used in this study were prepared by expression in *E. coli*, therefore contamination with *E. coli* LPS might account for the observed effect of HSP 60s. To exclude this possibility, we heat-treated the HSP 60s (both chlamydial and human) and the control *E. coli* endotoxin. Bacterial lipopolysaccharides exhibit thermostability, while most proteins are thermolabile. Heat treatment of either

chlamydial or human HSP 60 abrogated their ability to induce E-selectin, ICAM-1, or VCAM-1, but did not alter the effects of *E. coli* endotoxin (Figs. 1 and 2, *bottom*; Table 1), thus indicating lack of appreciable contamination with LPS in the HSP 60 preparations.

Chlamydial and human HSP 60s induce IL-6 production by ECs, SMCs, and macrophages. IL-6 is an important mediator of the acute phase response and can increase plasma levels of fibrinogen, C-reactive protein (CRP), and serum amyloid A protein (SAA) (36). Increased fibrinogen levels might promote thrombosis on a preexisting atheroma, and levels of IL-6, CRP, and SAA correlate with prognosis in patients with unstable angina (37, 38), and with the risk of myocardial infarction in healthy subjects (39). Infection of human monocytes with *C. pneumoniae* induces the production of IL-6 (13). We therefore tested whether chlamydial or human HSP 60 can induce IL-6 expression in ECs, SMCs, and macrophages. After 24 hours, chlamydial and human HSP 60s significantly increased the expression of IL-6 by ECs (Fig. 3, *top*), SMCs (Fig. 4, *top*), and macrophages (Fig. 5, *top*), as compared with control levels. While inactivated *C. pneumoniae* did not increase IL-6 expression by ECs and SMCs, it elicited IL-6 production by monocyte-derived macrophages (Figs. 3–5, *top*). This apparent discrepancy might relate to the well-described phagocytic properties of macrophages, a characteristic not prominent in either ECs or SMCs. Interestingly, while *E. coli* LPS significantly increased IL-6 expression in ECs and macrophages, it did not affect IL-6 production by SMCs, further supporting the lack of LPS contamination in the preparations of either HSP 60. Moreover, heat treatment of

either chlamydial or human HSP 60 abrogated their ability to induce IL-6 on ECs (Fig.3, *bottom*), SMCs (Fig. 4, *bottom*), and macrophages (Fig. 5, *bottom*), but did not alter the effect of *E. coli* endotoxin.

Chlamydial and human HSP 60s trigger NF-κB activation. We have recently shown that either chlamydial or human HSP 60 induce expression of TNF-α in mouse macrophages (10), and we have extended these findings to human macrophages (Kol, A., *et al.*, unpublished observations). The present results show that either HSP 60 can also induce E-selectin, ICAM-1, VCAM-1, and IL-6. Because NF-κB transcriptional complexes contribute importantly to the inducible expression of the genes encoding E-selectin, ICAM-1, VCAM-1, IL-6, and TNF-α (23), we sought evidence that HSP 60s trigger NF-κB activation. In particular, because IL-6 production was induced by HSP 60s in all three cell types examined in this study, we chose to detect NF-κB activation by a gel-shift assay using an oligonucleotide probe that contains the NF-κB–binding site of the IL-6 promoter. HSP 60s did indeed activate NF-κB: a complex was evident by 30 minutes after stimulation and appeared maximal and sustained from two to six hours (Fig. 6). The time course of response to chlamydial or human HSP 60 resembled that of LPS, a well-known activator of NF-κB (23). Heat treatment of chlamydial or human HSP 60 nearly abolished their ability to activate NF-κB, whereas the same treatment did not affect the response to LPS, thus rendering highly unlikely the possibility that LPS contamination in our HSP 60 preparations accounted for the observed effect on NF-κB activation (Fig. 7). Formation of binding complexes from nuclear extracts of cells treat-
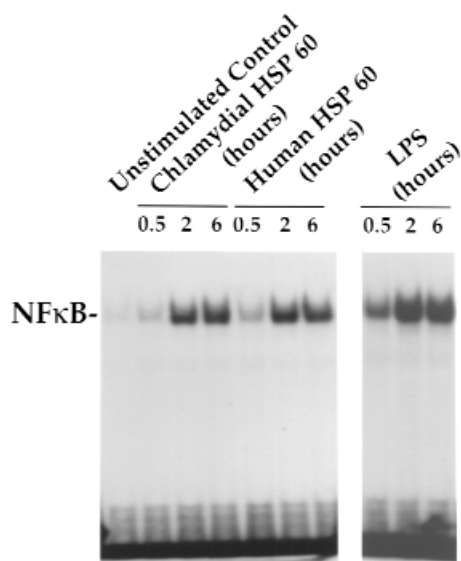
Figure 6
Chlamydial and human HSP 60s induce NF-κB activation in a time-dependent fashion. Endothelial cells were incubated with medium only (unstimulated control), or with chlamydial HSP 60 (2 μg/ml), human HSP 60 (2 μg/ml), or *E. coli* LPS (1 μg/ml) for 0.5, 2, and 6 h. Nuclear extracts were prepared and subjected to electromobility shift assay with an oligonucleotide containing the NF-κB–binding site of the IL-6 promoter. NF-κB complex formation was evident by 30 min and appeared maximal and sustained from 2 to 6 h. The time course of response to chlamydial or human HSP 60 was similar to that of LPS. *NF-κB*, nuclear factor-κB.
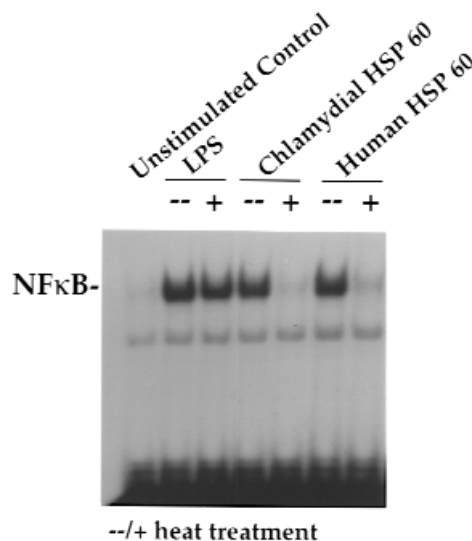
Figure 7
Chlamydial and human HSP 60s induce NF-κB activation: effect of heat treatment. Endothelial cells were incubated with medium only (unstimulated control), or with chlamydial HSP 60 (2 μg/ml), human HSP 60 (2 μg/ml), or *E. coli* LPS (1 μg/ml) for 2 h. A portion of each reagent was heat-treated before incubation by boiling for 20 min. Nuclear extracts were prepared and subject to electromobility shift assay with an oligonucleotide containing the NF-κB–binding site of the IL-6 promoter. Heat treatment abolished the induction of NF-κB complex formation by chlamydial and human HSP 60, but did not modify the effect of thermostable *E. coli* LPS.

ed with human HSP 60 were prevented by competition with unlabeled oligonucleotides containing the IL-6 or consensus NF-κB site, but not by an oligomer containing two point mutations in the IL-6 NF-κB site, verifying the specificity of the induced complexes for binding of NF-κB proteins (Fig. 8). Supershift analysis with a panel of anti-Rel antibodies identified p65 (Rel A) and p50 as components of human HSP–induced binding complexes, while antibodies to cRel, RelB, or nonimmune rabbit IgG did not supershift any complex (Fig. 9).

Discussion

The molecular mechanisms by which a chronic asymptomatic *C. pneumoniae* infection might contribute to atherogenesis and lesional complications remain obscure. This study demonstrated that chlamydial HSP 60, produced abundantly during chronic, persistent chlamydial infections, activates human vascular cell and macrophage functions related to atheroma formation and lesional complications.

We aimed to identify possible molecular effectors of *C. pneumoniae* contribution to atheroma. HSPs have been implicated as antigens stimulating autoimmunity in atherogenesis (40, 41). In addition, bacterial HSP 60, like another microbial product such as LPS, may activate the innate immune system (42). The findings of this study suggest a new function for chlamydial and also human HSP 60: the activation of intrinsic human vascular cells. The HSPs markedly increased E-selectin and ICAM-1 levels on endothelial cells and VCAM-1 to a much lesser extent. In contrast, on SMCs HSPs augmented neither ICAM-1 nor VCAM-1 expression. These distinct patterns may reflect differences in culture conditions between

HUVECs and SMCs. However, IL-6 increased in all cell types (HUVECs, SMCs, and monocyte-derived macrophages). Therefore, the differences in the regulation of adhesion molecule expression may also reflect cell type–specific control mechanisms. In either case, the expression of adhesion molecules by the endothelium likely contributes to leukocyte recruitment during atherogenesis, while the function of these molecules in smooth muscle cells remains unclear (17, 18, 43).

HSPs (or chaperonins) are generally considered to act intracellularly to preserve cellular protein stability in response to conditions such as heat shock, nutrient deprivation, infections, and inflammatory reactions (44). Our findings have particular interest, as they delineate a new possible function for this class of proteins, particularly HSP 60. The ability of either chlamydial or human HSP 60 to activate human vascular cells and to trigger NF-κB activation suggests a novel amplification loop in arterial inflammation. Chronic, persistent *C. pneumoniae* infection could provoke the expression of both chlamydial and human HSP 60s in the arterial wall. Both of these proteins could then amplify the ongoing inflammatory process, through their actions on human vascular cells described here.

Because HSPs usually localize within cells, they require release into the extracellular space to activate vascular cells. In this regard, it is well known that Chlamydiae, during their life cycle, undergo both phases of chronic, persistent, nonlytic infection, in which they remain viable, but do not replicate, and phases of lytic infection (15). During these lytic phases, the host cells release both their own HSP 60, produced during the previous chronic phase of infection, and also the human HSP 60, which has been produced in the host cell in response to the
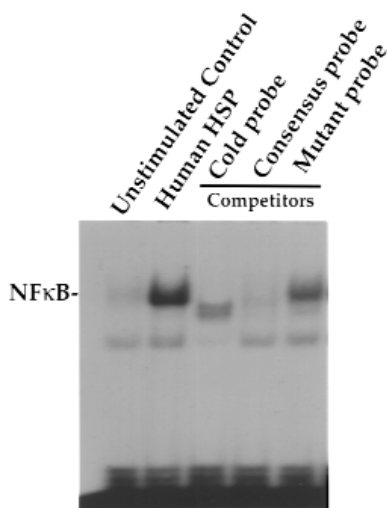
Figure 8
Human HSP 60 induces NF-κB activation: specificity of DNA-binding complexes. Endothelial cells were incubated with medium only (unstimulated control), or with human HSP 60 (2 μg/ml) for 2 h. Nuclear extracts were prepared and subject to electromobility shift assay with an oligonucleotide containing the NF-κB–binding site of the IL-6 promoter. Competition of DNA-binding activity in the presence of excess unlabeled competitor, consensus, or mutant NF-κB oligonucleotides demonstrate specificity of HSP 60–induced NF-κB DNA-binding complexes.
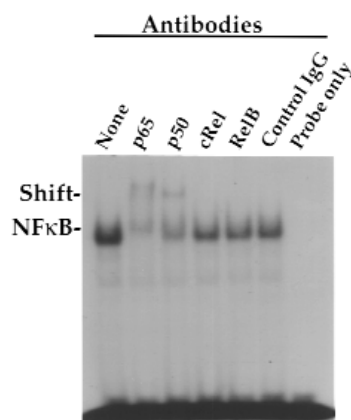
Figure 9
Human HSP 60 induces NF-κB activation: identification of p65 and p50 Rel proteins as components of DNA-binding complexes. Endothelial cells were incubated with medium only (unstimulated control), or with human HSP 60 (2 μg/ml) for 2 h. Rel proteins were identified by performing electromobility shift assay on nuclear extracts, with an oligonucleotide containing the NF-κB–binding site of the IL-6 promoter, in the presence of the indicated Rel antisera or with nonimmune rabbit IgG. NF-κB and supershifted complexes are indicated on the left: p65 (Rel A) and p50 were identified as components of human HSP–induced binding complexes, while antibodies to cRel, RelB, or nonimmune rabbit IgG did not shift any binding complex. *Probe only* indicates electromobility shift assay performed without nuclear extract.

infection and to previous noninfectious stimuli (44), and which we and others have shown in human atheroma (10, 21). In addition, several observations support the occurrence of cell death within atheroma (45, 46), providing another pathway for release of HSPs from cells.

In conclusion, this study demonstrates that either chlamydial or human HSP 60 activates human vascular cell functions relevant to atherogenesis and lesional complications. These findings contribute to our understanding of the molecular mechanisms by which a chronic asymptomatic chlamydial infection might influence atherogenesis and trigger acute events.

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