Oxidized LDL promotes the mitogenic actions of Chlamydia pneumoniae in vascular smooth muscle cells

Mirna N. Chahine1,2, Justin Deniset1,2, Elena Dibrov1,2, Satoru Hirono1,2, David P. Blackwood1,2, Jose Alejandro Austria1,2, and Grant N. Pierce1,2,3*

1Institute of Cardiovascular Sciences, St Boniface Hospital Research Centre, 351 Tache Ave., Winnipeg, Manitoba, Canada R2H 2A6; 2Department of Physiology, Faculty of Medicine, University of Manitoba, Winnipeg, Manitoba, Canada; and 3Faculty of Pharmacy, University of Manitoba, Winnipeg, Manitoba, Canada

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Aims
The atherogenic actions of Chlamydia pneumoniae (C. pneumoniae), a common respiratory pathogen, are dependent upon a high-cholesterol environment in vivo. It is possible that oxidized low-density lipoprotein (oxLDL) is responsible for promoting the atherogenic effects of C. pneumoniae through a stimulation of cell proliferation. This study determined whether oxLDL can enhance the mitogenic action of C. pneumoniae in vascular smooth muscle cells (VSMCs) and the involvement of mitogen-activated protein kinase (MAPK) pathways and heat shock protein 60 (HSP60) in these mechanisms.

Methods and results
Primary rabbit VSMCs were treated with live C. pneumoniae, heat-inactivated C. pneumoniae or infection medium, and subsequently incubated for up to 48 h in the presence or absence of oxLDL. Chlamydia pneumoniae infection alone stimulated cell proliferation and the addition of oxLDL significantly amplified this proliferative effect. This proliferation was accompanied by extracellular signal-regulated kinase-1 and -2 (ERK1/2) activation and an up-regulation of HSP60 expression. Changes in proliferation and HSP60 expression were attenuated by the inhibition of ERK1/2.

Conclusion
These results indicate a novel role for oxLDL in promoting the mitogenic actions of C. pneumoniae in the vasculature. ERK1/2 is an important factor in the stress-mediated response and HSP60 up-regulation in VSMC. These data provide mechanistic evidence that C. pneumoniae may stimulate atherogenesis.

Keywords
Infection • Oxidized low-density lipoprotein • Heat shock protein • Mitogen-activated protein kinase • Atherosclerosis

1. Introduction
Cell proliferation and inflammation are important components of atherosclerosis.1–3 Both cell proliferation and the inflammatory pathways in atherosclerosis may be initiated by a number of factors including C. pneumoniae a gram-negative, intracellular bacterium.4–6 The atherogenic effect of C. pneumoniae infection in vivo has been shown to be highly sensitive to a cholesterol-rich environment.4 However, the identity of the factors within that high-cholesterol environment that may have promoted the atherogenic actions of C. pneumoniae remains to be clearly elucidated. Oxidized LDL (oxLDL) is thought to be a potent atherogenic factor. It is an important component within a high-cholesterol environment and identified in plasma samples from patients with clinically relevant coronary artery disease.7 It is possible that the atherogenic action of C. pneumoniae may have been augmented through the presence of oxLDL in that cholesterol-rich environment. The purpose of the present study, therefore, was to investigate if oxLDL could potentiate the proliferative action of C. pneumoniae on vascular smooth muscle cells (VSMCs) and to determine the mechanism responsible for this interaction. Chlamydia pneumoniae can induce the proliferation of VSMCs.2–5 Similarly, oxLDL can induce transcription factors,8,9 and stimulate DNA synthesis and cell proliferation.3,10,11 These effects of oxLDL on cell growth correlate well with what we currently know about the molecular mechanisms whereby C. pneumoniae induces VSMCs proliferation. It is possible, therefore, but currently unknown, if oxLDL may...
augment the proliferative actions of *C. pneumoniae* on VSMCs and thereby contribute to plaque formation. Our results here demonstrate that oxLDL can potentiate the proliferative effects of *C. pneumoniae* in VSMCs and it achieves this through an activation of the mitogen-activated protein kinase (MAPK) pathway and induction of heat shock protein 60 (HSP60) expression.

## 2. Methods

### 2.1 Vascular smooth muscle cells

Smooth muscle cells were isolated from the thoracic aorta of male New Zealand white rabbits (*Oryctolagus cuniculus*) by an explant technique, as described previously. The animal use was according to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85–23, revised 1996). Approval was obtained for the conduct of this study from the Animal Care Protocol and Review Committee of the University of Manitoba. Cells were seeded directly into the wells in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% foetal bovine serum (FBS) overnight at a density of $5 \times 10^{3}$ per well for cell proliferation assay purposes or at $10^5$ cells per well for western blot analysis.

### 2.2 Chlamydia pneumoniae propagation and purification

*Chlamydia pneumoniae* AR39 strain was obtained from the University of Washington, Seattle, WA, USA. The organism was propagated as described previously. The titre of *C. pneumoniae* was determined by a colourimetric enzyme assay (CellTiter 96 Cell Proliferation Assay; Promega Corporation) (OD at 500 nm) based on a cytotoxicity assay.

### 2.3 Plasma lipoprotein isolation and oxidation

LDL (density 1.019–1.063 g/mL) was isolated by sequential ultracentrifugation from the plasma of male albino New Zealand White rabbits fed a 0.5% cholesterol-supplemented diet for a minimum of 8 weeks as described previously. Minimally modified LDL was oxidized with a Fe–ADP free radical-generating system. In a typical experiment, 1 mg/mL of LDL was incubated at 37°C for 3 h with freshly prepared 0.05 mM Fe and 0.5 mM ADP in sterile filtered 150 mM NaCl, pH 7.4, as described previously.

### 2.4 Exposure of VSMCs to *C. pneumonia*, oxLDL, and MAPK inhibitors

VSMCs were incubated with *C. pneumoniae* in DMEM (10% FBS) for 2 h. Control cells were maintained in an identical medium containing either a heat-inactivated *C. pneumoniae* (mock infected) or a medium free of *C. pneumoniae*. These cells were then incubated with 10 or 50 μg naLDL or oxLDL/mL for various time points (12, 24, and 48 h) in DMEM (0.1% FBS). Control cells were maintained in an identical medium without added naLDL or oxLDL. OxLDL-treated VSMCs, naLDL-treated VSMCs, or control VSMCs were incubated with specific extracellular signal-regulated kinase-1 and -2 (ERK1/2) (PD98059, 1 μM), p38 (SB203580, 1 μM), JNK (SP600125, 1 μM), and toll-like receptor (TLR) 2/4 [1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphoryl-choline (OxPAKC), 30 μg/mL] inhibitors for 48 h. These inhibitor concentrations used were not cytotoxic to non-treated VSMCs.

### 2.5 Cell proliferation assay

VSMCs seeded in 96-well plates were incubated with *C. pneumoniae* and/or oxLDL in DMEM (0.1% FBS). After 48 h, the number of living cells was determined by a colourimetric enzyme assay (CellTiter 96 Cell Proliferation Assay; Promega Corporation) (OD at 500 nm) based on a cytotoxicity enzyme activity present in viable cells.

### 2.6 Western blot analysis

Cells were harvested by trypsinization and counted using a haemocytometer. Cells (5 x 10⁴ per lane) were washed with PBS and lysed in sample buffer. Proteins were separated on a 9% SDS–polyacrylamide gel and transferred electrophoretically onto a nitrocellulose membrane as described previously. After blocking with blocking reagent, membranes were incubated with primary antibodies to proliferating cell nuclear antigen (PCNA, Sigma, St Louis, USA), p44/p42, phospho-p44/p42, p38, phospho-p38, SAPK/JNK, phospho-SAPK/JNK (Cell Signalling, Beverly, USA), HSP60 (StressGen Biotechnologies Corp), and to smooth muscle α-actin (Sigma, St Louis, USA) that was used as a loading control. HRP-conjugated anti-mouse IgG was used as a secondary antibody and bands were visualized on Fluor-S MAX (Bio-Rad Laboratories) with SuperSignal West Pico or Femto Chemiluminescent Substrates (Pierce, Rockford, IL, USA). Expression level of proteins was quantified using Quantity One program (Bio-Rad Laboratories).

### 2.7 Adenoviral vectors

Recombinant adenovirus-expressing human HSP60 (Ad-HSP60) was constructed using the AdEasy Vector System (Qbiogene) as reported previously. The viral particles were produced in QBI-293A cells and titre of the viral stock was determined by the tissue culture infectious dose method (TCID₅₀). A successful transfer of a reporter gene into 100% of VSMCs was achieved with a MOI (multiplicities of infection) of 100–200 viral particles/cell after 48 h incubation in DMEM/0.5% FBS. MOI 100 was the minimal concentration with the maximal transfer of the reporter gene into 100% of VSMCs. VSMCs were infected with Ad-HSP60 in DMEM/0.5% FBS for 48 h and cell numbers were determined by direct cell counting or by CellTiter 96 assay. A recombinant adenoviral construct that encodes green fluorescent protein (Ad-GFP) was used as a control.

### 2.8 Statistical analysis

Results were reported as mean ± SEM, analysed by one-way ANOVA followed by a Duncan’s multiple range post hoc test and *P* < 0.05.
considered statistically significant. Pearson correlation and linear regression analyses were used to assess the relationship between HSP60 expression levels and PCNA expression and cell number.

2.9 Reagents and chemicals
DMEM, FBS, and trypsin-EDTA were purchased from GIBCO-BRL. Cholesterol diet was purchased from Purina Test Diets (Richmond, IN, USA). Transferrin, selenium, ascorbate, insulin, cholesterol oxidase, and cholesterol esterase were obtained from Sigma-Aldrich (Oakville, ON, Canada). PD98059, SB203580, and SP600125 were purchased from Calbiochem (San Diego, CA, USA). OxPAPC was purchased from Invivogen (San Diego, CA, USA).

3. Results
3.1 OxLDL and C. pneumoniae induce VSMCs proliferation independently through ERK1/2 activation
Consistent with previous data from our laboratory,5 C. pneumoniae infection stimulated VSMCs proliferation after 48 h of incubation in a concentration-dependent manner (Figure 1). Heat-inactivated C. pneumoniae was used as a control (mock infection) and did not have any affect on cell number. In addition, VSMCs were exposed to 10 and 50 μg/mL of native LDL (naLDL) or oxLDL for 48 h.

Figure 2 Synergistic effect of oxLDL and C. pneumoniae on cell proliferation. Western blot analysis of PCNA expression (A) and cell number were determined (B) in VSMCs exposed to 10 μg/mL oxLDL and/or C. pneumoniae (1 × 10^5 IFU/mL) for 48 h. Even sample loading was confirmed by smooth muscle α-actin (SM-actin) detection (n = 4–5). Changes in the absorbance are expressed as a percentage of the mean value of control cells (n = 8). OD, optical density; Cpn, C. pneumoniae; PD, PD98059; SB, SB203580; SP, SP600125. * and †P < 0.05, † vs. control; values are means ± SEM.
VSMCs number was not affected by 10 μg/mL of naLDL (101 ± 2%) or oxLDL (105 ± 2%) but was significantly increased (106 ± 2% and 112 ± 1%) compared with control after exposure to 50 μg/mL naLDL and oxLDL, respectively (Figure 1). The main objective of this study was to determine whether oxLDL enhanced C. pneumoniae-induced VSMCs proliferation. To focus on this effect without the confounding influence of a proliferative action of oxLDL on its own, subsequent experiments were focused upon concentrations of naLDL or oxLDL that did not induce VSMCs proliferation by itself (10 μg/mL). The oxLDL was added to C. pneumoniae (1 × 10^5 IFU/mL) or mock-infected cells as described in Methods.

3.2 The combination of oxLDL and C. pneumoniae has an additive effect on induction of cell proliferation through a ERK1/2-dependent mechanism

PCNA was used as a marker for entry of the cell into the cell cycle. Exposure of VSMCs to oxLDL (10 μg/mL) for 48 h resulted in only a modest change, whereas C. pneumoniae (1 × 10^5 IFU/mL) infection induced a significant increase (65% change) in PCNA expression (Figure 2A). When these treatments were administered in combination, a further induction in PCNA expression (127% change) was observed (Figure 2A). These changes in PCNA expression in response to C. pneumoniae alone or in combination with oxLDL were stunted by ERK1/2 inhibition (PD98059) but remained unaffected with p38 (SB203580) and JNK (SP600125) inhibition (Figure 2A). In addition, TLR-2 and -4 inhibition did not alter C. pneumoniae or C. pneumoniae combined with oxLDL induced changes in PCNA expression (data not shown).

As PCNA was used as a marker for the cell cycle activation, cell proliferation was measured to determine whether the cell cycle was completed. The combination of C. pneumoniae infection and oxLDL induced a larger and significant increase (117%) in cell number compared with the other three treatments (Figure 2B). Inhibition of the ERK1/2 pathway using PD98059 normalized the increase in cell number in C. pneumoniae-infected cells and also in VSMCs exposed to the combination treatment (Figure 2B). Furthermore, the increases in cell number, induced by the two proliferative treatments, remained unchanged by TLR-2 and -4 inhibition with OxPAPC (Figure 2C).

MAPK activation is a major pathway involved in cell survival and proliferation and was assessed through p44/42, p38, and JNK phosphorylation levels (Figure 3A). p44/42 phosphorylation followed a similar trend as PCNA expression levels with no change to oxLDL treatment, an initial increase in activation with C. pneumoniae infection alone and a more pronounced activation when C. pneumoniae infection and oxLDL were administered in concert that was significant than all other treatments (Figure 3B). In contrast, p38 and JNK phosphorylation (Figure 3C and D) remained unchanged between treatments. These results demonstrate that C. pneumoniae and oxLDL act synergistically on VSMCs proliferation via an ERK1/2 MAPK pathway-dependent mechanism that is independent of TLR-2 or -4 activation.
3.3 *C. pneumoniae* infection enhances the expression of endogenous HSP60 in oxLDL-treated cells through ERK1/2 activation

We also investigated the possibility that the effects of *C. pneumoniae* and oxLDL may be achieved through another complimentary mechanism besides ERK activation. Previously, we established that *C. pneumoniae* can increase VSMCs proliferation through an induction of endogenous HSP60 expression. Could the same mechanism be involved in synergistic effects of oxLDL and *C. pneumoniae*?

Exposure of VSMCs to 10 μg/mL oxLDL and 1 × 10⁵ IFU/mL *C. pneumoniae* induced a significant time-dependent increase in both p44/42 MAPK expression and HSP60 expression (Figure 3A). After 48 h of treatment naLDL (10 μg/mL) or mock infection did not induce a significant change in HSP60 expression (data not shown). OxLDL (10 μg/mL) also did not induce a significant change in HSP60 expression at this time point (Figure 4A). However, *C. pneumoniae* significantly increased HSP60 expression and, more importantly, oxLDL potentiated this stimulation when the two were administered together (Figure 3B). Again, naLDL (10 μg/mL) did not significantly augment the effects of *C. pneumoniae* on its own (data not shown). The addition of PD98059 prevented HSP60 over-expression in *C. pneumoniae*-infected cells with or without oxLDL (Figure 3B). The addition of SB203580 or SP600125 had no effect on endogenous HSP60 expression (data not shown). Therefore, the increased endogenous HSP60 expression mediated by *C. pneumoniae* infection and oxLDL treatment appears to be achieved through an ERK1/2 MAPK pathway-dependent mechanism.

![Figure 4](https://academic.oup.com/cardiovascres/article-abstract/92/3/476/268702)
Importantly, the results obtained for HSP60 expression (Figure 3) were qualitatively similar to the results shown in Figure 2B for cell proliferation. These observations led us to hypothesize that HSP60 could constitute a critical component of the synergistic and mitogenic effects of C. pneumoniae infection and oxLDL treatment. Recombinant Ad-HSP60 was used in order to over-express HSP60 in the VSMCs. Over-expression of HSP60 was determined by western immunoblotting using the anti-mammalian HSP60 antibody (data not shown) as was established previously. Adenovirus carrying the enhance green fluorescent protein (Ad-EGFP) was used as a control. PCNA expression (Figure 5A) and cell proliferation (Figure 5B) was significantly stimulated in Ad-HSP60-infected VSMCs (123.1 ± 2.4) compared with Ad-EGFP-infected (101.9 ± 1.5) or uninfected (100.0 ± 1.1) control cells. Addition of 1 μM PD98059 to Ad-HSP60-infected VSMCs prevented the increase in cell proliferation (102.8 ± 3.3) which, once again, indicated the involvement of an ERK1/2 MAPK pathway-dependent mechanism. Ad-HSP60-infected VSMCs exhibited a similar degree of mitogenic effect (123.1 ± 2.4) as the one induced by oxLDL and C. pneumoniae infection (120.5 ± 1.3). HSP60 expression and cell proliferation in terms of PCNA expression (Figure 5A) and cell numbers (Figure 5B) were also tested for a correlative association. A significant correlation was observed between HSP60 and PCNA expression levels (n = 19, r = +0.79, P < 0.0001) (Figure 5A). A significant correlation was also observed between HSP60 expression levels and cell number (n = 31, r = +0.71, P < 0.0001) (Figure 5B). In addition, the positive slope of the regression lines calculated by Pearson’s correlation coefficient suggested that HSP60 and PCNA expression levels or HSP60 expression levels and cell number increase together. Indeed, 62.25% of the variance is shared between HSP60 and PCNA expression levels (r^2 = 0.6225) (Figure 5A) and 51.08% of the variance is shared between HSP60 expression levels and cell number (r^2 = 0.5108) (Figure 5B).

**Figure 5** Correlation between HSP60 expression levels and PCNA expression levels (A) or cell number (B). (A) PCNA and HSP60 expression were determined by western blotting in VSMCs exposed to 10 μg/mL oxLDL and/or C pneumoniae (1 × 10^5 IFU/mL) for 48 h or infected with adenoviral constructs; n = 4 different experiments. (B) Cell number was determined in VSMCs exposed to 10 μg/mL oxLDL with C. pneumoniae (1 × 10^5 IFU/mL) for 48 h or infected with adenoviral constructs. Changes in the absorbance are expressed as a percentage of the mean value of control cells; n = 7–8 different experiments. OD, optical density; Ad-HSP60, adenoviral constructs encoding HSP60; Cpn, C pneumoniae; r^2 = coefficient of determination.
4. Discussion

VSMCs proliferation is an important component of the developing atherosclerotic plaque.\textsuperscript{2,3} Chlamydia pneumoniae infection induced VSMCs proliferation, as shown previously.\textsuperscript{5,16,17} Exposure to a lower concentration of oxLDL (10 \(\mu\)g/mL) did not induce an increase in cell number (Figures 1 and 2B), whereas treatment with a higher concentration of oxLDL (50 \(\mu\)g/mL) significantly induced cell proliferation (Figure 1). This is consistent with previous data.\textsuperscript{1} We chose to use the lower concentration of oxLDL to clearly identify if without confounding effects of its own it could further enhance VSMCs proliferation induced by C. pneumoniae infection. Although treatment with oxLDL (10 \(\mu\)g/mL) alone did not affect VSMCs propagation, when combined with C. pneumoniae infection, it could further enhance the C. pneumoniae-induced cell proliferation. Significant increases in cell proliferation and PCNA expression were not observed in control settings [mock infection and/or naLDL (10 \(\mu\)g/mL) treatment] (Figure 1). These results suggest that the synergistic effects are specific to treatment with oxLDL.

Other investigators have examined the cytotoxic effects of the combined treatment of oxLDL and C. pneumoniae on different cells. For example, a synergistic effect of C. pneumoniae and oxLDL increased the expression of adhesion molecules (ICAM-1, E-selectin) in endothelial cells,\textsuperscript{18} induced markers of cell death in macrophages,\textsuperscript{19} and shifted oxLDL-induced cytotoxicity towards necrosis.\textsuperscript{19} The cytotoxicity may have occurred because they used much higher concentrations of oxLDL (100 and 200 \(\mu\)g/mL)\textsuperscript{20} or because macrophages are more susceptible to apoptosis in response to oxLDL.\textsuperscript{19} In our study, we used minimally oxLDL\textsuperscript{1,15} in lower concentrations (10 \(\mu\)g/mL) that are thought to be more physiologically relevant.\textsuperscript{1,15}

The molecular mechanism whereby oxLDL augmented C. pneumoniae-induced VSMCs proliferation in the present study clearly involved a specific activation of the ERK1/2 MAPK pathway. Previous work has demonstrated p44/p42 MAPK activation by C. pneumoniae infection in fibroblasts,\textsuperscript{21} endothelial cells,\textsuperscript{22,23} and VSMCs.\textsuperscript{17} In our study, C. pneumoniae infection significantly increased the levels of phosphorylated p44/42 MAPK. Blocking the pathway with PD98059 inhibited these increases. The present study demonstrates that oxLDL is capable of augmenting the activation of the p44/42 MAPK pathway beyond that observed by C. pneumoniae alone.

TLR signalling has been suggested to play an important role in pathogen/stress-mediated proliferative responses in VSMCs.\textsuperscript{17,24} Sasu et al. demonstrated that C. pneumoniae infection (3 \(\times\) 10\textsuperscript{7} IFU/mL) induced proliferation in human VSMCs was partially mediated by extracellular chlamydial HSP60 (cHSP60) through a TLR-4 dependent pathway.\textsuperscript{17} In addition, soluble recombinant human HSP60 has been demonstrated to be proliferative in venous smooth muscle cells through TLR-2 and -4 signalling.\textsuperscript{24} In the current study, TLR-2 and -4 signalling inhibition did not influence the proliferative status of C. pneumoniae-infected VSMCs with or without oxLDL treatment. These data suggest that both the individual and synergistic proliferative responses to C. pneumoniae infection and oxLDL are not mediated through a cHSP60 and/or host HSP60 autocrine/paracrine effect. This result is in agreement with our previous work\textsuperscript{5} which noted that extracellular administration of cHSP60 was not proliferative in rabbit primary VSMCs.

The involvement of endogenous HSP60 in the oxLDL-mediated augmentation of VSMCs proliferation is more difficult to demonstrate conclusively. Chlamydia pneumoniae-induced HSP60 expression was enhanced by oxLDL more than that demonstrated by C. pneumoniae alone. Furthermore, HSP60 expression correlated significantly with PCNA expression and cell number (Figure 5). Adenoviral-induced over-expression of HSP60 stimulated VSMCs proliferation and this mitogenic effect was quantitatively comparable with the one induced by combined treatment with oxLDL and C. pneumoniae. These results are all consistent but do not definitively prove an involvement of HSP60 in the augmentation of the proliferative action of C. pneumoniae that was enhanced by oxLDL. The effects of oxLDL/ C. pneumoniae on HSP60 expression may also be associated with the ERK1/2 MAPK pathway, p44/42 MAPK activation correlated.
with HSP60 protein expression (Figure 4A). Furthermore, a selective MEK1/2 inhibitor, PD98059, prevented over-expression of HSP60 (Figure 4B) and the cell proliferation induced by HSP60. The present study suggests, therefore, that a p44/p42 MAPK-dependent mechanism may be involved in HSP60 expression and in the synergistic effects of oxLDL and C. pneumoniae on VSMCs proliferation (Figure 5). Unfortunately, deletion of HSP60 from VSMCs with an RNAi approach has proved to be difficult to interpret due to cytotoxic side effects associated with the depletion so we cannot conclusively address its involvement in the effects of oxLDL beyond evidence by association (Figure 6).

Previous studies have shown that a high-cholesterol environment was necessary in vivo for C. pneumoniae-induced atherogenesis in the LDLr−/− mouse.6,7 Our data demonstrate that oxLDL, a well-recognized component of a high-cholesterol environment,7 can enhance the atherogenic action of C. pneumoniae by promoting VSMCs proliferation. Thus, our data has identified one component of the high-cholesterol environment that may be responsible for stimulating the atherogenic action of C. pneumoniae. These results may have clinical implications. Antibiotic treatment of patients with cardiovascular disease who have shown evidence of prior exposure to C. pneumoniae infection has yielded inconsistent results to date in a number of trials.25–27 It is possible that the populations studied were not ideal. Focusing C. pneumoniae antibiotic trials on a patient population with high-cholesterol/oxLDL levels may yield more consistent, positive results. Furthermore, our data raise the intriguing possibility that ERK1/2 MAPK and HSP60 may be central factors in the progression of proliferative atherogenic lesions during inflammatory challenges.

Conflicts of interest: none declared.

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