GroEL1, a Heat Shock Protein 60 of Chlamydia pneumoniae, Induces Lectin-Like Oxidized Low-Density Lipoprotein Receptor 1 Expression in Endothelial Cells and Enhances Atherogenesis in Hypercholesterolemic Rabbits

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GroEL1, a Heat Shock Protein 60 of Chlamydia pneumoniae, Induces Lectin-Like Oxidized Low-Density Lipoprotein Receptor 1 Expression in Endothelial Cells and Enhances Atherogenesis in Hypercholesterolemic Rabbits

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Lectin-like oxidized low-density lipoprotein receptor 1 (LOX-1) plays a major role in oxidized low-density lipoprotein-induced vascular inflammation. Chlamydia pneumoniae has been found in atherosclerotic lesions and is related to atherosclerotic pathogenesis, although its specific mechanism remains unknown. This study was conducted to investigate the mechanisms of LOX-1 expression in GroEL1 (a heat shock protein from C. pneumoniae)-administered human coronary artery endothelial cells (HCAECs) and atherogenesis in hypercholesterolemic rabbits. We demonstrated that in the hypercholesterolemic rabbit model, GroEL1 administration enhanced fatty streak and macrophage infiltration in atherosclerotic lesions, which may be mediated by elevated LOX-1 expression. In vitro study using HCAECs, stimulation with GroEL1 increased TLR4 and LOX-1 expression. Increased LOX-1 expression was downregulated by Akt activation and PI3K-mediated endothelial NO synthase activation. PI3K inhibitor and NO synthase inhibitor induced LOX-1 mRNA production, whereas the NO donor ameliorated the increasing effect of LOX-1 mRNA in GroEL1-stimulated HCAECs. LOX-1 expression was regulated by NADPH oxidase, which mediates reactive oxygen species production and intracellular MAPK signaling pathway in GroEL1-stimulated HCAECs. Treatment with polyethylene-glycol–conjugated superoxide dismutase, apocynin, or diphenylene iodonium significantly decreased GroEL1-induced LOX-1 expression, as did the knockdown of Rac1 gene expression by RNA interference. In conclusion, the GroEL1 protein may induce LOX-1 expression in endothelial cells and atherogenesis in hypercholesterolemic rabbits. The elevated level of LOX-1 in vitro may be mediated by the PI3K–Akt signaling pathway, endothelial NO synthase activation, NADPH oxidase-mediated reactive oxygen species production, and MAPK activation in GroEL1-stimulated HCAECs. The GroEL1 protein of C. pneumoniae may contribute to vascular inflammation and cardiovascular disorders. The Journal of Immunology, 2011, 186: 4405–4414.

Chlamydia pneumoniae is a Gram-negative bacterium that has a biphasic elementary body (EB) and reticulate body life cycle. There is extensive evidence indicating that C. pneumoniae may play a key role in the development of atherosclerosis and coronary artery disease. Clinical evidence has shown that early fatty streaks present accumulation of oxidized LDL (oxLDL)-mediated atherogenesis. We demonstrated that C. pneumoniae accelerates the formation of complex atherosclerosis in ApoE3-Leiden mice (4) and can induce increased uptake of low-density lipoprotein (LDL) in macrophages (5). Although C. pneumoniae can induce LDL oxidation within the neointima (6) and may potentially increase the development of hypercholesterolemia-mediated atherosclerosis, there is no direct evidence of C. pneumoniae infection preceding oxidized LDL (oxLDL)-mediated atherogenesis.

During the infectious process of C. pneumoniae in target cells, EBs require both attachment to and phagocytosis by host cells

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(7). Heat shock protein 60 of C. pneumoniae (GroEL) is also expressed on the surface of EBs, and the proteins could be fallen off from the EBs. Reports have demonstrated that the EB surface-associated GroEL1 is the major binding adhesion protein and plays an important role in the pathogenesis of infectious diseases (8).

In clinical practice, the GroEL1 protein indeed causes several complicated diseases, including respiratory tract diseases and vascular diseases (9). Additionally, the GroEL1 protein may initiate the secretion of IL-6 and TNF-α in dendritic cells, and stimulate production of IL-1β, -6, and -8 and proliferation in vascular cells and mononuclear cells by triggering intracellular signaling pathway activation within eukaryotic cells (10–12).

One crucial event for C. pneumonia-induced atherosclerosis is believed to be oxLDL accumulation, which results in the formation of foam cells. C. pneumonia infection enhances lectin-like oxidized LDL receptor 1 (LOX-1), but not scavenger receptor expressed by endothelial cell (SREC) expression (13), which accelerates atherogenesis in the presence of hypercholesterolemia. We hypothesized that C. pneumonia GroEL1 protein may increase LOX-1 expression in the endothelium, which mediates oxLDL uptake and the development of serious atherosclerosis. Therefore, we also examined whether GroEL1 increased neointimal hyperplasia and LOX-1 expression in hypercholesterolemic rabbits. Furthermore, we explored the cellular events and underlying mechanisms involved in GroEL1-induced LOX-1 expression using human coronary artery endothelial cells (HCAECs) in vitro.

Materials and Methods

Production and purification of C. pneumonia elementary bodies

C. pneumonia (TWART-TW-183) were cultured on Hela 229 cells, and 100 ml inoculum from ~70°C storage was used in HEPES/sucrose/citrate dilution. Plates were centrifuged 1 h at 70 × g to enhance cellular attachment. The supernatant was aspirated and discarded, and chlamydial growth media was added. Plates were incubated at 36°C with CO2 for 3 d, and then infected cells were harvested. The harvested cells were sonicated for 15 s, then centrifugated at 700 × g for 15 min, and then pelleted at 18,000 × g for 30 min. The pellet was resuspended in HEPES-sucrose-cation solution. The 3–5 ml suspension was layered over a 13 ml HEPES-sucrose-cation adding 7 ml Renograin and centrifuged 18,000 × g at 30°C for 1 h. The final suspension containing EBs was to the original volume in 0.01 M PBS.

Construction of C. pneumoniae GroEL expression vectors

The genomic DNA of C. pneumonia was extraction from EBs using EasyPure Genomic DNA mini kit (Biomann Scientific, Taipei, Taiwan). Segment containing the open reading frame of the GroEL was originally PCR amplified by using 100 ng C. pneumonia genomic DNA as template, 0.2 m camera DNTPs, 1 μM each of gene-specific primers, and 1 U PFU DNA polymerase (Promega, Madison, WI) with the following program: 1 cycle of 95°C for 5 min, 38 cycles of 95°C for 45 s, 68°C for 45 s, and 72°C for 2 min; 1 cycle of 68°C for 45 s and 72°C for 10 min; and a final incubation at 72°C for 10 min with 1 U Taq DNA polymerase. The following gene-specific primers were used in the PCR reaction: GroEL Pr-forward, 5'-CGAATTCTTAAGGAGAACAACGATGGCAG-3' (forward primer contained an EcoRI site) and GroEL Pr-reverse, 5'-CGAATTCTTAAGGAGAACAACGATGGCAG-3' (forward primers contained an Eagl site). The amplified GroEL cDNA fragment was then cloned into pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA), and subsequently cloned in-frame into the EcoRI and NotI sites of pGEX-5X-1 expression vector (GE Healthcare Amersham Biosciences) for expression in Escherichia coli.

Purification of GroEL recombinant protein

BL21 cells were transformed with pGEX-5X-1-GroEL expression vector and GroEL recombinant proteins were purified. Briefly, the BL21 cells containing pGEX-5X-1-GroEL plasmid were grown overnight at 37°C in 2 ml Luria-Bertani medium supplemented with 100 μg/ml ampicillin. Then, 1.25 ml overnight culture was transferred into 100 ml Luria-Bertani ampicillin medium and grown at 37°C to an A600 of 0.6–0.8 (~2 h). Fusion protein expression was then induced by adding isopropyl β-D-thiogalactoside to a final concentration of 1 mM at 30°C for 6 h. Bacteria were pelleted by centrifugation for 10 min at 8000 rpm, and rGroEL was extracted under native conditions according to the protocol of GST Gene Fusion System manufacturer’s instructions (GE Healthcare Amersham Biosciences). Finally, rGroEL protein was purified by elution buffer containing 50 mM Tris-HCl and 10 mM reduced glutathione (pH 8.0). The quantity of rGroEL protein was measured using the Bio-Rad protein assay (Bio-Rad, Hercules, CA). The fusion protein was detected by SDS gel electrophoresis, and identified by immunoblotting with a GST Ab (GE Healthcare Amersham Biosciences). Endotoxin in the rGroEL protein were measured using a Limulus amebocyte lysate kit from Cambrex. LPS levels were below 1 pg/ml.

Measurement of GroEL protein cytotoxicity and activity

Cell cytotoxicity of rGroEL protein was analyzed by the MTT assay. HCAECs were grown in 96-well plates and incubated with various concentrations (1–500 ng/ml) of rGroEL for 24 h. Subsequently, 0.5 μg/ml MTT was added to each well, and incubation was continued at 37°C for an additional 4 h. DMSO was added to each well, and the absorbance was recorded at 530 nm using a DIAAS Microplate Reader (Dynex Technologies). The activity of rGroEL was measured by ELISA. Human mononuclear cell line THP-1 cells were seeded in 24-well plates at a density of 105 cells/ml/well, and these were then treated with various concentrations of GroEL protein (1–100 ng/ml) for 1 or 3 h. The culture medium was collected to quantify the levels of TNF-α and IL-6 by using the DusSet ELISA development kits (R&D Systems), and the absorbance was recorded using a DIAAS Microplate Reader (Dynex Technologies).

Animal experiment

All animals were treated according to protocols approved by the Institutional Animal Care Committee of the Taipei Medical University (Taiwan). Experimental procedures and animal care conformed to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (Publication 85-23, revised 1996). Fifteen adult male New Zealand White rabbits (2.5–3 kg) were used. After 1 wk on a commercial rabbit chow diet (Scientific Diet Services, Essex, U.K.) at 60 g/kg/d with water ad libitum, nine animals were placed on a 2% high-cholesterol (HC) diet (Purina Mills, St. Louis, MO) and six animals were placed on a normal diet. The animals were divided into five groups, as follows: group 1 was the control; group 2 was fed the HC diet; group 3 received the HC diet and i.v. injections of GroEL1 (2 μg/kg body weight) through the ear vein at the end of weeks 2, 3, and 4; group 4 received the HC diet and i.v. injections of GroEL1 (4 μg/kg body weight) at the end of weeks 2, 3, and 4; and group 5 received the normal chow diet and GroEL1 (4 μg/kg body weight) at the end of weeks 2, 3, and 4. The animals were sacrificed and the abdominal aortas were then harvested, gently dissected free of adherent tissues, rinsed with ice-cold PBS, and incubated with 4% buffered paraformaldehyde, paraffin embedded, and then cross-sectioned for morphology (H&E staining) and immunohistochemistry. The thoracic aortas were also collected and stained with Sudan IV solution for visualization of the fatty streak areas.

Measurement of erythrocyte sedimentation rate and serum C-reactive protein

Erythrocyte sedimentation rate (ESR) and serum C-reactive protein (CRP) levels were analyzed to evaluate GroEL1-induced systemic inflammatory responses. Arterial blood was collected from the ear artery into tubes containing sodium citrate. ESR is used primarily to detect occult processes and monitor inflammatory conditions. Rabbit ESR was measured by the Westergren method. CRP is regarded as an acute-phase reactant in the serum in a wide variety of diseases. Rabbit serum was separated from whole blood, and CRP was then measured in triplicate with a commercial ELISA kit (Immunochemistry Consultants Laboratory).

Biochemical measurements

Blood samples for biochemical measurements were collected from each animal before and at 2 and 5 wk of the experiment. Samples were separated by centrifugation, and the serum was stored at ~80°C until analysis. Serum total cholesterol and triglyceride were measured using Merck assay kits (Darmstadt, Germany). Serum blood urea nitrogen (BUN), creatinine, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were also measured using a SPOTCHEM™ automatic dry chemistry system (SP-4410; Arkay, Shanghai, Japan).

Sudan IV staining of arteries for fatty streaks

The fixed thoracic aortas were immersed in 70% ethanol for 30 min and then in the 1% Sudan IV/70% ethanol solution at room temperature for 2 h. The
samples were then digested in 70% ethanol for 3 min and rinsed with running tap water. Sudanophilic areas were measured quantitatively by computer-assisted planimetry, and the extent of the lesions was expressed as a proportion of the total surface area (surface area of lesions/total surface area of the thoracic aorta).

Cell culture

HCAECs were purchased from Cascade Biologics (Portland, OR). Human monocytic THP-1 cells were purchased from American Type Culture Collection (Manassas, VA). Cell cultures and passages were performed according to the manufacturer’s instructions. HCAECs were used at passages 3–8. Purity of the HCAEC cultures was verified by immunostaining with a mAb directed against smooth muscle-specific α-actin (R&D Systems, Minneapolis, MN).

Immunohistochemical and immunofluorescent staining

Immunohistochemical and immunofluorescent staining were performed on serial 5-μm-thick paraffin-embedded sections of rabbit abdominal aortas and coverslip-grown HCAECs using anti–LOX-1, anti–SREC, scavenger receptor B1 (SR-B1), or anti–RAM-11 Abs. DAPI was used to identify the nucleus. The slides were observed with microscopy or confocal microscopy.

Uptake of 1,1'-dioctadecyl-3,3',3'-tetramethyl-indocarbocyanine perchlorate–LDL by HCAECs

Human LDL (d:1.019–1.063 g/ml) was isolated by sequential ultracentrifugation of fasting plasma samples from healthy adult males (14). The native LDL was oxidized, as described by Steinbrecher et al. (15, 16). The oxLDL was labeled with 1,1'-dioctadecyl-3,3',3'-tetramethyl-indocarbocyanine perchlorate (DiI), as described previously (17). To examine cellular uptake of oxLDL, HCAECs were seated on culture slides and incubated for 4 h in serum-free medium. The cells were then washed with PBS, mounted on coverslips, and examined with confocal microscopy.

Western blot analysis

Membrane fractions and total cell lysates were extracted from HCAECs. Proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane. The membranes were probed with mouse anti–LOX-1 (Santa Cruz Biotechnology), anti–SREC (Santa Cruz Biotechnology), anti–SR-B1 (Santa Cruz Biotechnology), anti–endothelial NO synthase (eNOS), Millipore), anti–phospho-eNOS (Millipore), anti–Akt (Anaspec), anti–phospho-Akt (Merck, Darmstadt, German), goat anti-TLR2 Ab (R&D Systems), rabbit anti–p38, rabbit anti–phospho-p38, rabbit anti–stress-activated protein kinase (SAPK)/JNK, rabbit anti–phospho–SR-B1 (Santa Cruz Biotechnology), anti–endothelial NO synthase (eNOS), Millipore), anti–phospho-eNOS (Millipore), anti–Akt (Anaspec), anti–phospho-Akt (Merck, Darmstadt, German), goat anti-TLR2 Ab (R&D Systems), goat anti-TLR2 Ab (R&D Systems), goat anti-phospho-SAPK/JNK, rabbit anti–p44/42 MAPK, or mouse anti–phospho-p44/42 MAPK Abs. DAPI was used to identify the nucleus. The slides were observed with microscopy or confocal microscopy.

Quantitative real-time PCR

Total RNA was isolated using TRizol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer’s instructions. LOX-1 and SREC mRNA expression was determined by quantitative real-time PCR. The level of LOX-1 and SREC mRNA expression was determined in arbitrary units by comparison with an external DNA standard, which was amplified with the primers for LOX-1 and SREC. LOX-1 and SREC mRNA expression was determined in arbitrary units by comparison with an external DNA standard, which was amplified with the primers for LOX-1 and SREC.

Knockdown gene expression with interference RNA

Intracellular TLR2 and TLR4 expression was knocked down by transfection with interference RNA (small interfering RNA [siRNA]). Cells (10⁶) were trypsinized and resuspended in 100 ml Nucleofector solution (Amaza Biosystems), and 30 nM TLR2 or TLR4 siRNA (Ambion Catalog 4635) was used for knockdown validation.

NADPH oxidase activity assay

NADPH oxidase activity was determined with superoxide-dependent lucigenin chemiluminescence, as previously described (18). The 40 μg membrane protein extraction and 5 μM dark-adapted lucigenin were added to a 96-well luminometer plate and adjusted to a final volume of 250 μl with oxidase assay buffer before 100 μM NADPH was added. Relative light units were read with a luminometer (Dynatech ML2250; Dynatech Laboratories). Light emission was recorded every minute for 15 min and was expressed as mean relative light units/min.

Pull-down assay for Rac1 activity

Rac1 activation was measured using a GST-(p21-activated kinase)-p21 binding domain (GST-[PAK]-PBD) fusion protein, which binds to activated Rac1. HCAECs were lysed in lysis buffer (25 mM HEPES, 150 mM NaCl, 1% Igepal CA-630, 10% glycerol, 10 mM MgCl₂, 1 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin). The supernatant was collected and incubated with GST-(PAK)-PBD fusion protein. The protein–bead complexes were then recovered by centrifugation and washed. Following the last wash, the protein–bead complexes were resuspended in SDS reducing sample buffer and resolved by 12% SDS-PAGE. The proteins were transferred to polyvinylidene difluoride membrane, and the membrane was incubated with mouse anti-Rac1 Ab (Upstate Biotechnology) and HRP-conjugated secondary Ab. Activated Rac1 was then detected using an ECL detection kit.

Statistical analyses

Values are expressed as means ± SEM. Statistical evaluation was performed using Student’s t test and one- or two-way ANOVA, followed by Dunnett’s test. A p value <0.05 was considered significant.

Results

High concentrations of GroEL1 induce THP-1 cell TNF-α production and HCAEC cytotoxicity

Treatment of HCAECs with 1, 10, 25, 50, 100, or 250 ng/ml GroEL1 protein for 24 h did not result in cell viability (Supplemental Fig. 1A). In contrast, a high concentration (500 ng/ml) of GroEL1 protein may cause a significant reduction in cell viability. Treatment of THP-1 cells with 100 ng/ml GroEL1 for 1 h and 1–100 ng/ml GroEL1 for 3 h may induce TNF-α production (Supplemental Fig. 1B). The results indicated that rGroEL1 has bioactivity and results in cell cytotoxicity at high concentrations.

Biocchemical measurements for rabbits

During the experimental period, weight gain and final weight did not differ significantly between the groups of animals (data not shown). As shown in Table I, serum AST, ALT, BUN, and creatinine levels also showed no significant difference between groups. Serum total cholesterol levels were increased after 2 wk HC diet. At the end of the fifth experimental week, the HC diet may have the elevated total cholesterol level. Compared with the control group, GroEL1 protein treatment did not increase the serum total cholesterol level. Additionally, serum triglyceride levels did not increase significantly in any of the experimental group during the experimental period.

GroEL1 protein induces inflammatory responses in rabbits

ESR and serum CRP levels were analyzed to monitor GroEL1-induced systemic inflammation (Table II). In the control and HC diet groups, ESR and CRP levels did not change during the experiment. However, ESR increased in the GroEL1 groups at weeks 4 and 5. CRP levels increased at week 3 and continued to increase throughout the experimental period. During the experimental period, heart rate, rectal body temperature, and respiratory rate of the rabbits were monitored. This result suggests that GroEL1 protein

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The Journal of Immunology 4407
injection may keep rabbits in a nonsepsis state and induce chronic combining acute systemic inflammation.

**GroEL1 enhances atherosclerotic lesion formation in HC diet-fed rabbits**

Representative photographs of the fatty streaks of thoracic aortas stained with Sudan IV from the five groups are shown in Fig. 1A. There were no atherosclerotic lesions in the aortas of control rabbits. Sudanophilic atherosclerotic lesions in the HC diet group were slightly increasing under en face observation. A significant area of the aortic intimal surface from the HC diet plus 2 µg/kg body weight (BW) GroEL1 group and the HC diet plus 4 µg/kg BW GroEL1 group was covered with fatty streaks. Furthermore, use of 4 µg/kg BW GroEL1 induced more serious fatty streak formation than use of 2 µg/kg BW GroEL1 in rabbits fed an HC diet. In contrast, administration of 4 µg/kg BW GroEL1 protein did not increase atherosclerotic lesion formation in rabbits fed a normal chow diet.

Fig. 1B shows that rabbits in the control, HC diet, and 4 µg/kg BW GroEL1 groups did not have thickened intima or atherosclerotic lesion formation in the abdominal aortas. Abdominal aortas had slight lesion formation in the HC diet plus 2 µg/kg BW GroEL1 group and marked lesion formation in the HC diet plus 4 µg/kg BW GroEL1 group compared with these areas in the control group. Staining with anti–RAM-11 Ab for identification of infiltrated macrophages showed that fewer macrophages infiltrated into the vessel walls in the control, HC diet, and 4 µg/kg BW GroEL1 groups compared with the HC diet plus GroEL1 groups (Fig. 1C). These results demonstrate that administration of GroEL1 protein significantly increased macrophage infiltration and atherosclerotic plaque formation in HC diet-fed rabbits.

**LOX-1 expression in GroEL1-administered rabbits**

Immunohistochemical staining was performed using Abs against LOX-1, SREC, and SR-B1 on sections of the abdominal aortas (Fig. 1D). Compared with sections from the control group, GroEL1 administration slightly enhanced LOX-1 expression in the endothelium of the HC diet group, whereas strong LOX-1 staining was seen on the luminal surface of the 4 µg/kg BW GroEL1 group and in the neointima of the HC diet plus 4 µg/kg BW GroEL1 group. In contrast, neither HC diet nor GroEL1 administration induced SREC and SR-B1 expression in the abdominal aorta in rabbits. These results demonstrate that GroEL1 administration increased LOX-1 expression and significantly severe atherosclerotic lesion formation in the aorta.

**GroEL1 induces LOX-1 expression and enhances DiI-oxLDL uptake in HCAECS**

A DiI-oxLDL uptake assay was performed to investigate whether oxLDL uptake is enhanced in GroEL1-stimulated endothelial cells.

### Table I. Plasma biochemical characteristics (n = 6) in experimental rabbits

<table>
<thead>
<tr>
<th>Weeks</th>
<th>Control</th>
<th>HC Diet</th>
<th>GroEL1 4 µg/kg BW</th>
<th>HC Diet and GroEL1 Treatment</th>
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<td>2 µg/kg BW</td>
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<td>BUN (mg/dl)</td>
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Values are mean ± SD. *p < 0.05 compared with 0 wk at the same group.

### Table II. Inflammatory characteristics (n = 6) in experimental rabbit

<table>
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<th>Weeks</th>
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<th>HC Diet and GroEL1 Treatment</th>
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<td>0.88 ± 0.03</td>
<td>0.83 ± 0.12</td>
<td>0.91 ± 0.74</td>
<td>1.17 ± 0.53</td>
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<td>3</td>
<td>1.32 ± 0.48</td>
<td>1.05 ± 0.42</td>
<td>18.05 ± 0.78</td>
<td>13.48 ± 1.03</td>
</tr>
<tr>
<td>4</td>
<td>2.32 ± 1.08</td>
<td>1.53 ± 0.52</td>
<td>11.05 ± 0.89</td>
<td>10.99 ± 1.10</td>
</tr>
<tr>
<td>5</td>
<td>1.36 ± 0.32</td>
<td>1.90 ± 0.14</td>
<td>19.83 ± 1.29</td>
<td>11.90 ± 0.91</td>
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Values are mean ± SD. *p < 0.05 compared with control group at the same time point. **p < 0.05 compared with 0 wk at the same group.
Confocal microscopy demonstrated that treatment with GroEL1 protein for 24 h significantly increased DiI-oxLDL uptake in HCAECs (Fig. 2A). Real-time PCR demonstrated that GroEL1 treatment for 12 h may induce LOX-1 mRNA expression, but not SREC or SR-B1 mRNA expression (Fig. 2B). Immunofluorescence also showed that treatment with GroEL1 protein for 24 h induces LOX-1 protein expression in HCAECs (Fig. 2C). LOX-1, SREC, and SR-B1 were originally identified on the membrane of vascular endothelial cells; Western blotting showed that GroEL1 protein increases intracellular LOX-1 production and elevates membrane LOX-1 expression in HCAECs (Fig. 2D).

TLR4 mediates LOX-1 expression in GroEL1-stimulated HCAECs

C. pneumonia GroEL1 may induce innate immune responses in vitro via TLR2 and TLR4 (19). As shown in Fig. 3A, GroEL1 protein significantly induced TLR4 expression, but not TLR2 expression. Treatment with 100 ng/ml GroEL1 for 12 h significantly induced LOX-1 mRNA expression (Fig. 3B). Addition of a mouse anti-human TLR (hTLR)4 Ab (10 μg/ml) and subsequent incubation for 30 min or transfection with 25 nM TLR4 siRNA prior to GroEL1 treatment significantly reduced LOX-1 mRNA production in HCAECs. As a negative control in the competition assay, a nonspecific IgG2a isotype Ab was substituted for the TLR4-specific Ab; however, the nonspecific Ab and the silencer-validated siRNA did not affect GroEL1-induced LOX-1 mRNA production (data not shown). In contrast, Fig. 3C shows that mouse anti-hTLR2 Ab and TLR2 siRNA did not block LOX-1 mRNA production in GroEL1-stimulated HCAECs, suggesting the GroEL1-induced LOX-1 expression in HCAECs is mediated by TLR4.

Akt signaling pathway and eNOS activity may be involved in GroEL1-induced LOX-1 expression

We next investigated the effects of GroEL1 protein on Akt kinase signals and eNOS expression in HCAECs. After 6 h of incubation, Akt phosphorylation was significantly decreased in GroEL1 protein-cultured HCAECs (Fig. 4A). Furthermore, eNOS phosphorylation at Ser1177 was significantly decreased both in GroEL1 protein-stimulated and LY294002 (a PI3K inhibitor)-treated HCAECs compared with control conditions (Fig. 4B). The PI3K–Akt signaling pathway did not involve eNOS production in GroEL1-stimulated HCAECs (LY294002 treatment did not decrease eNOS production; Fig. 4B); the potential roles of MAPK-related mechanisms were also examined. Pretreatment with SB203580 (p38 MAPK inhibitor), SP600125 (a JNK/SAPK inhibitor), or PD98059
(an MEK1 antagonist) for 1 h did not ameliorate the inhibitory effects of GroEL1 protein on eNOS phosphorylation (Fig. 4C). Interestingly, GroEL1-stimulated decreases in eNOS production were reduced by SB203580, but not by SP600125 or PD98059. This result probably indicated that GroEL1 protein influences eNOS production mediating by p38 MAPK. The potential roles of PI3K/Akt and eNOS in LOX-1 expression in GroEL1-stimulated HCAECs were then examined. Coincubation with NO donor S-nitrosocysteine (SNOC) significantly ameliorated the increases in LOX-1 mRNA (Fig. 4D) and LOX-1 protein (Fig. 4E) on GroEL1-stimulated HCAECs. In contrast, incubation with NO synthase inhibitor t-N^2-nitro-l-arginine methyl ester (t-NAME) or PI3K inhibitor LY294002 significantly enhanced LOX-1 expression. These data indicate that GroEL1 protein may upregulate LOX-1 expression in endothelial cells by modulating PI3K/Akt-, eNOS-, and p38 MAPK-related mechanisms.

**GroEL1-induced LOX-1 expression is mediated by Rac1 and NADPH oxidase activation and ROS generation**

To determine whether GroEL1 protein induces NADPH oxidase activity in HCAECs, HCAECs were treated with 100 ng/ml GroEL1 protein for 0 (●), 60 (○), 90 (▲), or 120 (△) min, and the membrane fraction was then assayed for NADPH oxidase activity (Fig. 5A). GroEL1 protein treatment resulted in a time-dependent increase in enzyme activity. Diphenylene iodonium (DPI; 100 μM) was used to determine NADPH oxidase activity (Fig. 5A), which is associated with Rac1 activation insofar as it has been reported to play important roles in NADPH oxidase activation (20). To further examine whether GroEL1-induced NADPH oxidase activation is accompanied by increases in Rac1 activation, we measured activated Rac1 with a GST-(PAK)-PBD fusion protein pull-down assay. HCAECs were treated with 25–100 ng/ml GroEL1 protein for 120 min. Total cell lysates were extracted and measured for Rac1 activity. GroEL1 treatment rapidly induced Rac1 activation in a dosage-dependent manner (Fig. 5B). To explore whether NADPH-mediated reactive oxygen species (ROS) are involved in GroEL1-induced LOX-1 expression, HCAECs were pretreated with various antioxidants or transfected with Rac1 siRNA prior to stimulation with GroEL1 protein. As shown in Fig. 5C, GroEL1 or H2O2 significantly induced LOX-1 mRNA expression, which was significantly blocked by pretreatment with polyethylene-glycol–conjugated superoxide dismutase (a permeable antioxidant enzyme), apocynin (a specific NADPH oxidase inhibitor), or DPI for 1 h. GroEL1-induced LOX-1 mRNA expression was completely blocked by Rac1 siRNA, but not by the negative control siRNA. These results suggest that GroEL1-induced LOX-1 expression is mediated by an oxidative stress-related mechanism and that the NADPH oxidase subunit Rac1 plays a critical role in LOX-1 regulation.

**Intracellular MAPK signaling in GroEL1-induced LOX-1 expression**

GroEL1 protein markedly induced the phosphorylation of MAPKs, including p38 MAPK, ERK1/2, and SAPK/JNK (Fig. 5D), after exposure to 25–100 ng/ml GroEL1 protein for 30 min. DPI significantly decreased SAPK/JNK activation in GroEL1-stimulated HCAECs, but did not influence p38 MAPK or ERK1/2 activation (Fig. 5D), suggesting that NADPH-oxidase–derived ROS are involved in SAPK–JNK signaling pathway activation. Real-time PCR demonstrated that GroEL1-induced LOX-1 mRNA expression was reduced by SB203580 (a p38 MAPK inhibitor), SP600125...
The role of GroEL1 in the induction of LOX-1 expression in HCAECs is mediated by TLR4 and TLR2. 

**FIGURE 3.** GroEL1-induced LOX-1 expression in HCAECs is mediated by TLR4. A, TLR4 and TLR2 protein expression was analyzed. B, HCAECs were pretreated with anti-hTLR4 Ab or transfected with TLR4 siRNA prior to GroEL1 treatment. LOX-1 mRNA expression was analyzed by real-time PCR. C, HCAECs were pretreated with anti-hTLR2 Ab or transfected with TLR2 siRNA prior to GroEL1 treatment. LOX-1 mRNA expression was analyzed by real-time PCR. Data represent the results of three independent experiments (mean ± SEM). *p < 0.05.

(a SAPK/JNK inhibitor), or SB203580 plus SP600125, but not by PD98059 (a MEK1 antagonist) (Fig. 5E). These results suggest that SAPK/JNK and p38 MAPK play more significant roles than ERK1/2 in the GroEL1-mediated LOX-1 mRNA expression transcriptional regulatory signaling pathway.

**Discussion**

In the current study, to our knowledge, we demonstrated for the first time that *C. pneumoniae* GroEL1 protein may enhance LOX-1 expression in endothelium and mediate oxLDL uptake and atherosclerosis progression. In fact, GroEL1 increased neointimal hyperplasia and LOX-1 expression in hypercholesterolemic rabbits. Furthermore, our in vitro findings suggest that GroEL1 interacts with TLR4 and plays critical roles in oxLDL uptake of endothelial cells, which results from elevated LOX-1 expression and is mediated by NADPH oxidase activation, PI3K/Akt-mediated eNOS activation, and the MAPK signaling pathways.

**C. pneumonia infection and atherogenesis**

The role of *C. pneumoniae* infection in the pathogenesis of atherosclerosis remains controversial (21–24). Meta-analyses of randomized clinical trials of antibiotic therapy for secondary prevention of coronary heart disease did not show any benefit (25, 26). There was also no effect of possible *C. pneumoniae* infection on serological markers. Although these results do not conclusively rule out a role of *C. pneumoniae* in the pathogenesis of atherosclerosis, they definitively do not support the use of antibiotics in the secondary prevention of coronary heart disease. Our data showed that *C. pneumoniae* GroEL1 protein alone did not induce fatty streak formation, but did enhance the formation of atherosclerotic lesions in HC diet-fed rabbits, suggesting the potential role of *C. pneumoniae* heat shock proteins in atherosclerosis induction in the presence of hypercholesterolemia. This may provide a potential rationale for the dramatic failure of clinical trials using antibiotics for atherosclerosis.

Studies have shown associations between atherosclerosis and different pathogens, and early investigations showed the presence of infectious pathogens in the whole arterial vessel tree (27). It is known that pathogens such as *C. pneumoniae* can induce macrophage foam cell formation (28), an effect that might be increased if an individual has been infected by multiple pathogens (5). TLR/MyD88 and liver X receptor signaling pathways reciprocally control *C. pneumoniae*-induced acceleration of atherosclerosis (29).

It has been hypothesized that the infectious pathogen contains proteins that are homologous to parts of the host proteins, resulting in an immune response called infection-induced molecular mimicry (30). It has recently been shown that *C. pneumoniae* heat shock protein 60 localizes in human atheroma and regulates macrophage TNF-α and matrix metalloproteinase expression (31); in fact, this heat shock protein 60 can activate human vascular endothelium, smooth muscle cells, and macrophages (10), and can stimulate cellular LDL oxidation in vitro (6). Our data show that administration of GroEL1 protein significantly increased macrophage infiltration in atherosclerotic plaques in HC diet-fed rabbits, supporting the results that genital *Chlamydia* infections are associated with significant induction of the chemokines and chemokine receptors that are involved in the recruitment of immune cells into infection sites (32).

The hypercholesterolemic with GroEL1 administration rabbit model was used in the current study. Rabbits were injected with 2 or 4 µg/kg body weight GroEL1, which represents a GroEL1 level of 50 or 100 ng/ml plasma, respectively. The combined results of ESR and serum CRP measurements are a useful indicator of inflammation. The rectal body temperature and respiratory rate of the rabbits were kept in the normal range (the normal range of rectal body temperature, 38–40°C and respiratory rate, 30–60 breaths/min) after GroEL1 was administered. Elevated CRP and ESR, but the maintenance of normal vital signs, indicated that the dose of GroEL1 was sufficient to produce inflammation in the animals, which remained in a nonseptic state. Indeed, we decided the dose of GroEL1 in the in vivo study with 25–100 ng/ml was consistent with the pathophysiological state of hypercholesterolemia and GroEL1-administrated rabbit study in vivo. Previous findings have demonstrated that a repertoire of LOX-1 is associated with atherosclerotic lesions. Although atherosclerotic plaque lipids can be found in macrophages and foam cells, and LOX-1 is not only expressed by endothelial cells, but also by smooth muscle cells and macrophages, we therefore cannot exclude that macrophages/smooth muscle cells via the GroEL1/LOX-1 route play some roles during atherogenesis. Further studies are required to clarify the interaction between GroEL1 and LOX-1 expression in smooth muscles and monocytes.

**C. pneumonia and LOX-1 expression**

The histopathological features of *C. pneumoniae*-induced aortic lesions closely resemble early changes produced by a diet enriched with low amounts (0.15%) of cholesterol in rabbits, which resulted in serum cholesterol levels (4.1 mM) similar to what is recom-
C. Pneumoniae GroEL1 Induces LOX-1

In the current study, exogenous addition of GroEL1 induced oxLDL uptake and LOX-1 expression in endothelial cells. TLR4 was the mediator in GroEL1-induced LOX-1 expression. These results suggest that the GroEL1-induced expression of LOX-1 in HCAECs is mediated by TLR4. This result is inconsistent with the observation that the mitogenic effect of C. pneumoniae on vascular smooth muscle cells could be mimicked by exogenous Chlamydia heat shock protein 60 via a TLR4-mediated signaling pathway (12). Several groups have reported that TLR4 expression is upregulated by bacterial LPS and certain cytokines in endothelial cells (40); extracellular heat shock protein 60 may still act as a signal transducer to stimulate cells via upregulated TLR4.

Regulation of LOX-1

Induced endothelial expression of LOX-1 upon various pathologic stimuli would provide a molecular link for incorporation of oxLDL into cells, resulting in cellular activation, dysfunction, and injury (41). Because oxLDL has been shown to generate ROS, tethering oxLDL via LOX-1 would focus oxidant stress on cellular targets, resulting in changes in gene expression and cellular phenotype and, in some cases, induction of endothelial dysfunction. Recent studies showed that oxLDL binding to LOX-1 in endothelial cells induces generation of superoxide anion (42). As DPI, a selective inhibitor of NADPH oxidase, drastically reduced intracellular superoxide concentration, oxLDL-induced superoxide generation might be related to increased NADPH oxidase activity. The increased intracellular superoxide anion accelerates NO inactiva-
The presence of oxLDL decreases intracellular NO concentrations in basal, bradykinin-stimulated, and thrombin-stimulated conditions. In addition, in HCAECs, oxLDL decreases PKB/Akt phosphorylation and downregulates eNOS activity. Our data also demonstrated that GroEL1 induced LOX-1 expression in the Akt–eNOS-related pathway, which suggests that the Akt–eNOS-related pathway plays an important role in GroEL1-induced LOX-1 activation.

Production of oxLDL promotes free radical generation and causes lipid peroxidation in endothelial cells. Regulation of LOX-1 gene expression is redox sensitive; therefore, ROS generated by GroEL1 in vascular cells may be key intermediates in the regulation of LOX-1 gene expression. Evidence linking oxidative stress with MAPK activation in vascular cells additionally supports these findings. In conclusion, our results demonstrated that the C. pneumoniae GroEL1 protein may induce LOX-1 expression in endothelial cells, which mediates fatty streak formation in atherogenesis. The elevated level of LOX-1 may be mediated by the PI3K–Akt signaling pathway, eNOS activation, NADPH oxidase-mediated ROS production, and MAPK activation in GroEL1-stimulated HCAECs. The C. pneumoniae GroEL1 protein may contribute to vascular inflammation and cardiovascular disorders. Our work identifying LOX-1 as a target gene for GroEL1 provides a basis for further investigation of LOX-1 modulation as a therapeutic strategy for atherogenesis in C. pneumoniae infection.

**FIGURE 5.** NADPH-oxidase–mediated ROS generation and MAPK signaling pathways are involved in LOX-1 expression. A, NADPH oxidase activity was measured with a superoxide-dependent lucigenin chemiluminescent assay. B, HCAECs were treated with GroEL1 protein. Cytosolic Rac1 activity was measured using a pull-down assay. C, HCAECs were pretreated with polyethylene-glycol–conjugated superoxide dismutase, apocynin (APO), or DPI, or were transfected with 25 nM Rac1 siRNA, followed by GroEL1 protein stimulation for 12 h. LOX-1 mRNA expression levels were analyzed by quantitative real-time PCR. H2O2 was used as the positive control. Silencer-validated siRNA (NC siRNA) was used for knockdown validation. D, HCAECs were treated with GroEL1 in the presence or absence of 100 μM DPI before the cell lysates were extracted. Phosphorylation of p38 MAPK, ERK1/2, and SAPK/JNK was analyzed by Western blotting. E, HCAECs were transfected with 25 nM Rac1 siRNA, followed by GroEL1 protein stimulation for 12 h. LOX-1 mRNA expression levels were analyzed by quantitative real-time PCR. Data represent the results of three independent experiments (mean ± SEM; *p < 0.05).
C. PNEUMONIAE GroEL1 INDUCES LOX-1

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Disclosures
The authors have no financial conflicts of interest.

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