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C-reactive protein/oxidized low density lipoprotein/β2-glycoprotein I complexes induce lipid accumulation and inflammatory reaction in macrophages via p38/mitogen-activated protein kinase and nuclear factor-κB signaling pathways

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Abstract. Oxidized low-density lipoprotein (oxLDL) can bind to β2-glycoprotein I (β2GPI) and C-reactive protein (CRP) to form stable complexes, which exert certain effects in diabetic cardiovascular disease. A previous study by our group has confirmed that the resulting complexes promote atherosclerosis in diabetic BALB/c mice. The present study was designed to investigate the effects and potential mechanisms of oxLDL complexes on lipid accumulation and inflammatory reactions in RAW264.7 macrophages cultured in a hyperglycemic environment. Cultured cells were divided into seven groups, which were treated with phosphate-buffered saline (control), CRP, β2GPI, oxLDL, CRP/oxLDL, oxLDL/β2GPI or CRP/oxLDL/β2GPI. The results revealed the formation of foam cells in the oxLDL, CRP/oxLDL, oxLDL/β2GPI as well as CRP/oxLDL/β2GPI groups. Compared with oxLDL, the three complexes induced less lipid accumulation ($P<0.05$) through inhibiting the expression of CD36 mRNA and promoting the expression of and ABCG1 mRNA ($P<0.05$ vs. oxLDL). Furthermore, the levels of inflammatory factors interleukin (IL)-1β, IL-6 and tumor necrosis factor-α were elevated in the CRP/oxLDL and CRP/oxLDL/β2GPI groups ($P>0.05$ vs. oxLDL), and obvious effects on p38/mitogen-activated protein kinase and nuclear factor (NF)-κB phosphorylation were also observed in these groups ($P<0.05$ vs. oxLDL). These results suggested that CRP/oxLDL/βG2P1 complexes may induce lipid accumulation and inflammation in macrophages via the p38/MAPK and NF-κB signaling pathways. However, some differences were observed between the complexes, which may be attributed to the property of each constituent; therefore, further studies are required.

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Introduction

Atherosclerosis (AS) is a multifactorial pathological process of arterial vasculature undergoing gradual intima thickening, causing decreased elasticity and narrowing. The causal association between AS and cholesterol metabolism is well established. The appearance of lipid accumulation and foam cells is a characteristic histological finding in early AS lesions (1,2).

Oxidized low-density lipoprotein (oxLDL) is highly proinflammatory and has a key role in the generation and progression of AS. OxLDL binds to β2-glycoprotein I (β2GPI) and C-reactive protein (CRP), resulting in the formation of covalently stable complexes, such as CRP/oxLDL, oxLDL/β2GPI and CRP/oxLDL/β2GPI. β2GPI is a highly glycosylated plasma protein, which serves as the main specific antigen for anti-phospholipid antibodies associated with atherothrombotic complications frequently observed in patients with systemic autoimmune diseases (3). OxLDL interacts with β2GPI via 7-ketocholesterol, which bears a w-carboxyl acyl chain, to form a covalent oxLDL/β2GPI complex (4,5). CRP is an acute-phase reactant that has a major role in innate immunity and inflammation. Multiple clinical studies have demonstrated the predictive value of increased CRP levels for atherothrombotic events, suggesting that CRP is a serological marker for AS (6). CRP is able to bind to oxLDL but not to native LDL

through the recognition of a phosphorylcholine moiety in oxLDL (7).

Accumulating evidence suggested the participation of these complexes in AS-associated processes (8,9). In auto-immune diseases, such as anti-phospholipid syndrome (APS), oxLDL/β2GPI and its antibody were found in the intima of AS lesions and the specific immune complexes were taken up avidly by macrophages via anti-β2GPI auto-antibody-mediated phagocytosis (8). However, in the serum of diabetics, anti-β2GPI auto-antibody levels were found to be low (10), while oxLDL/β2GPI, CRP/oxLDL and CRP/oxLDL/β2GPI complexes were present at high concentrations and were all positively correlated with the thickness of the intima media, a sensitive index for cardiovascular disease (11). Direct evidence for the involvement of CRP and β2GPI in AS was provided by immunohistochemical staining, which demonstrated the presence of CRP as well as β2GPI co-localized with oxLDL in human AS lesions (12). A previous study by our group reported that the CRP/oxLDL/β2GPI complex aggravated AS progression in diabetic BALB/c mice, which may be mediated through the p38/mitogen-activated protein kinase (MAPK) pathway (9). However, *in vitro*, the roles of these oxLDL complexes in the onset and development of AS in diabetes have not been elucidated. The present study aimed to investigate the impact of various oxLDL complexes on lipid accumulation and inflammatory reactions in RAW264.7 macrophages under hyperglycemic conditions.

Materials and methods

Materials and reagents. The RAW264.7 mouse macrophage cell line was purchased from the American Type Culture Collection (ATCC no TIB-71; Manassas, VA, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). oxLDL was purchased from XINYUANJIAHE Biotechnology Co., Ltd. (Beijing, China). CRP was purchased from ProSpec (cat no. pro-557; Rehovot, Israel). Oil Red O and hematoxylin were purchased from Sigma-Aldrich (St. Louis, MO, USA). An intracellular total cholesterol assay kit was purchased from Applygen Techonology Co., Ltd (Beijing, China). Interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α ELISA kits were purchased from Uscn Life Science Inc. (Hubei, China). The TRIzol reagent, RevertAid™ First Strand cDNA Synthesis kit and Micro Bicinchoninc Acid (BCA) Protein Assay kit were purchased from Invitrogen Life Technologies, Inc. (Carlsbad, CA, USA). SYBR® Premix Ex TaqTM DNA polymerase were purchased from Takara Bio. Inc. (Otsu, Japan). Rabbit monoclonal antibodies to p38/MAPK, phosphorylated (p)-p38MAPK (Thr180/Tyr182), nuclear factor (NF)-κB (p65) and p-NF-κB (Ser 276) were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Mouse monoclonal antibody to β-actin and horseradish peroxidase (HRP)-labeled goat anti-rabbit and anti-mouse immunoglobulin (Ig)G antibody were purchased from Tianjin Sungene Biotec Co., Ltd (Tianjin, China). High-performance chemiluminescence kit was purchased from Beijing ComWin Biotech Co., Ltd. (Beijing, China).

Purification of β2GPI. β2GPI was purified from normal human plasma as described previously (9). The blood was donated by a healthy voluntary blood donor at the Metabolic Diseases Hospital of Tianjin Medical University (Tianjin, China) in April 2014. The present study was approved by the ethics committee of Metabolic Diseases Hospital fo Tianjin Medical University. Written informed consent was obtained form the healthy blood donor. Plasma β2GPI was precipitated using 3% (v/v) perchloric acid (Sigma-Aldrich) and isolated by Heparin-Sepharose affinity chromatography (HiTrap Heparin; GE Healthcare, Little Chalfont, UK). Liquid Chromatography-Mass Spectrometric analysis (1100LC/MSD; Agilent Technologies, Santa Clara, CA, USA) was used to confirm this protein. The purity of β2GPI was confirmed by SDS-PAGE using a 10% Tris-glycine gel. The SDS-PAGE analysis of the protein sample showed an identical band to that of the standard β2GPI sample (Crystal Chem, Inc., Downers Grove, IL, USA). The purified β2GPI was tested to exclude the possibility of lipopolysaccharide contamination using the Limulus ES-II Test (13,14). Briefly, 250 μg human β2GPI was incubated with an extraction solution of chloroform/methanol (2:1) for 2 h at 25˚C with constant mixing on a rotator. Following centrifugation, the aqueous supernatant containing lipid-free β2GPI was retained. All treated preparations were sterilized using a 0.2 μm filter and tested for endotoxin activity using a commercial Limulus amebocyte lysate assay (Associates of Cape Cod, Inc., East Falmouth, MA, USA), according to the manufacturer's protocol. The lower limit of endotoxin detection of the LAL assay was 0.005 endotoxin units/ml. The BCA method was used to determine the concentration of β2GPI.

Complex preparation and identification. The preparation of CRP/oxLDL, oxLDL/β2GPI and CRP/oxLDL/β2GPI complexes was performed as described previously (9,11,15). Briefly, CRP/oxLDL and oxLDL/β2GPI complexes were pre-formed by incubating oxLDL [1 mg apolipoprotein B (apoB) equivalent/ml] and β2GPI (1 mg/ml) in the absence of $CaCl_2$, as well as oxLDL (1 mg apoB equivalent/ml) and CRP (1 mg/ml) in the presence of 2 mM $CaCl_2$, at 37˚C for 16 h. Subsequently, the purified oxLDL/β2GPI (1 mg/ml of apoB equivalent) was further incubated with CRP (0.1 mg/ml) in the presence of 2 mM $CaCl_2$ at 37˚C for another 16 h to form the covalent CRP/oxLDL/β2GPI complex. The identity of the oxLDL/β2GPI, CRP/oxLDL and CRP/oxLDL/β2GPI complexes was confirmed by ELISAs as described previously (9,11).

Cell culture. RAW264.7 macrophages were cultured in DMEM with 25 mmol/l glucose (Beijing Solarbio Science & Technology Co., Ltd.), which was supplemented with 10% (v/v) FBS, 100 U/ml penicillin and 100 g/ml streptomycin at 37˚C in a humidified incubator with 5% (v/v) CO_2. Cells were seeded onto six-well or 24-well cell culture plates at a density of $1x10^5$/ml cultured for 12 h and then serum-starved for another 12 h. The cells were then incubated for another 24 h in FBS-free hyperglycemic DMEM with the following intervention factors: phosphate-buffered saline (PBS; control), 80 μg/ml CRP, 80 μg/ml β2GPI, 80 μg/ml oxLDL, 160 μg/ml CRP/oxLDL, 160 μg/ml oxLDL/β2GPI or 176 μg/ml CRP/oxLDL/β2GPI, which all contained the same amount

of oxLDL. The concentrations and incubation time with the above reagents are based on those used in previous studies by our group (9,16). Following incubation, the culture medium was collected for ELISAs and the cells were washed twice with PBS prior to subsequent analysis.

Oil Red O staining. Oil Red O staining of foam cells was performed as described in a previous study by our group (16). Briefly, the macrophages were fixed in 10% (v/v) formaldehyde solution for 30 min. Fixed cells were washed with PBS and then with 60% (v/v) isopropanol solution twice for 5 min each. Next, the cells were stained with freshly prepared Oil Red O working solution for 30 min at 60˚C. The nuclei were lightly stained with hematoxylin for 5 min. Stained cells were washed with distilled water, mounted in neutral balsam (Beijing Solarbio Science & Technology Co., Ltd.) and then observed using an inverted microscope (DM4000 B; Leica Microsystems, Wetzlar, Germany).

Intracellular total cholesterol content assay. After treatment with the complexes, the culture medium was removed and cells were washed twice with ice-cold PBS. The cells were collected and sonicated at 4˚C in lysis buffer (1X Tris-buffered saline, pH 7.5, 10 mM EDTA, 1% Triton X-100, 10 mM NaF, 1 mM phenylmethanesulfonylfluoride, all Beijing Solarbio Science & Technology Co., Ltd.; and 1 mM sodium orthovanadate, Sigma-Aldrich). After homogenization, the supernatant was obtained by centrifugation (10,000 xg for 10 min at 4˚C). Protein concentrations were determined according to BCA assay. Total cholesterol levels were measured using a commercially available intracellular total cholesterol assay kit according to the manufacturer's instructions. Cholesterol levels were expressed as μmol per mg protein.

Total RNA isolation and reverse-transcription quantitative polymerase chain reaction (PCR). Primers for scavenger receptor B (CD36), scavenger receptor B1 (SRB1), adenosine triphosphate (ATP) binding cassette receptor A1 (ABCA1), ATP binding cassette receptor G1 (ABCG1) and β-actin mRNA were designed according to the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/) using Primer 5.0 (Premier Biosoft, Palo Alto, CA, USA) and Oligo 6.0 (Molecular Biology Insights, Inc., Colorado Springs, CA, USA) software. The primers were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). The primer sequences were as follows: CD36 forward, 5'-GAACCACTGCTTTCAAAAACTGG-3' and reverse, 5'-TGCTGTTCTTTGCCACGTCA-3'; SRB1 forward, 5'-TTTGGAGTGGTAGTAAAAAGGGC-3' and reverse, 5'-TGACATCAGGGACTCAGAGTAG-3'; ABCA1 forward, 5'-AGTGATAATCAAAGTCAAAGGCACAC-3' and reverse, 5'-AGCAACTTGGCACTAGTAACTCTG-3'; ABCG1 forward, 5'-TTCATCGTCCTGGGCATCTT-3' and reverse, 5'-CGGATTTTGTATCTGAGGACGAA-3'; β-actin forward, 5'-TGGAGAAGAGCTATGAGCTGCCTG-3' and reverse, 5'-GTGCCACCAGACAGCACTGTGTTG-3'.

Total RNA was extracted from cells using TRIzol reagent according to the manufacturer's instructions. RNA (2 μg) was reverse transcribed into cDNA using RevertAid™ First Strand cDNA Synthesis kit, as previously described (16). The PCR was performed using an iQ™ Real-Time PCR Detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). For qPCR, each reaction comprised 5 μl SYBR® Green II (Takara Bio. Inc.), 0.4 μl downstream/upstream primers, 1 μl cDNA, and 3.2 μl diethylpyrocarbonate-treated water. PCR cycling conditions were as follows: 50˚C for 2 min, 94˚C for 3 min, followed by 40 cycles at 94˚C for 30 sec, 60˚C for 30 sec and 72˚C for 20 sec. The relative quantification was based on β-actin to determine fold-differences in the expression of the target gene. The ΔΔCt-method was used for the normalization procedure. The final results are expressed as the $2^{-\Delta\Delta Ct}$ value between the experimental and the control group.

ELISA. RAW264.7 cells were seeded at $1x10^5$/well in a 24-well plate. After serum-starvation for 12 h, cells were treated with various reagents as described above. IL-1β, IL-6 and TNF-α were determined in the supernatants of the cells using commercially available ELISA kits according to the manufacturer's instructions. Standard samples provided in the kits were used to calculate absolute index levels. The protein levels of IL-1β, IL-6 and TNF-α in the cell culture media were expressed in pg/ml.

Western blot analysis. After treatment with the complexes as described above, the culture medium was removed and the reaction was terminated by adding 1 ml cold PBS containing 100 μM sodium vanadate (Sigma-Aldrich). The samples were then placed on ice, washed with ice-cold PBS and lysed in radioimmunoprecipitation (RIPA) lysis buffer for 30 min. Lysates were clarified by centrifugation at 12,000 x g for 15 min at 4˚C, and the protein content in the supernatant was measured using the BCA method. 10 μg protein was subjected to 12% SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was blocked in freshly prepared 5% skimmed milk in Tris-buffered saline/0.05% Tween 20 (TBST) for 2 h at room temperature (RT), subsequently incubated with primary antibodies against p38/MAPK (cat. no. 14451), p-p38/MAPK (Thr180/Tyr182; cat. no. 4511), NF-κB (p65) (cat. no. 8242), p-NF-κB (Ser 276) (cat. no. 3037) (all 1:1,000) or β-actin (cat. no. DKM9001; 1:5,000) diluted in 5% skimmed milk - TBST, overnight at 4˚C. Following three washes for 10 min each with TBST, the membranes were incubated with HRP-conjugated goat anti-rabbit (cat. no. LK2001) or anti-mouse (cat. no. LK2003) IgG in 5% skimmed milk - TBST (1:3,000) for 1 h at RT. The membrane was then extensively washed with TBST. The immunoreactive bands were detected using enhanced chemiluminescence reagents, blots were imaged using radiography with an ultraviolet transmission analyzer (GE Healthcare, Piscataway, NJ, USA) and images were captured onto film (Kodak, Inc., Rochester, NY, USA), and analyzed using BandScan software (Bio-Rad Gel Doc 2000; Bio-Rad Laboratories, Inc.). β-actin was used as the internal standard protein.

Statistical analysis. All experiments were performed at least three times in duplicate. SPSS 20.0 software (International Business Machines, Armonk, NY, USA) was used for statistical analysis. Values are expressed as the mean ± standard deviation. Comparisons among multi-groups were accomplished by one-way analysis of variance and differences between two groups were analyzed using the Student-Newman-Keuls Test.

P<0.05 was considered to indicate a statistically significant difference between values.

Results

OxLDL and its complexes induce lipid accumulation in macrophages. Incubation of RAW264.7 cells with oxLDL produced lipid-rich foam cells characterized by an intense red color accompanied with an increase in cell size and a decrease in cell count. Incubation of macrophages with CRP or β2GPI alone did not produce any observable effects; however, in the CRP/oxLDL, oxLDL/β2GPI and CRP/oxLDL/β2GPI groups, a considerably larger amount of red lipid droplets was observed in the cytoplasm (Fig. 1).

OxLDL and its complexes increase the intracellular total cholesterol content in RAW264.7 macrophages. Among all of the groups, the total cholesterol (TC) content in cells treated with oxLDL was highest, which was 6.7-fold that of the control group (P<0.05). Treatment with CRP or β2GPI alone produced no obvious change in the cholesterol content compared with that in the control group. The cholesterol accumulation induced by the three complexes significantly decreased in comparison to that in the oxLDL group (P<0.05). TC in the CRP/oxLDL group was 5.5-fold of that in the control group, which was higher than that in the oxLDL/β2GPI and CRP/oxLDL/β2GPI groups (P<0.05) and 3.1- and 4.0-fold of that in the control group, respectively. TC in the oxLDL/β2GPI group was lower than that in the CRP/oxLDL and CRP/oxLDL/β2GPI groups (P<0.05) (Table I).

oxLDL and its complexes increase CD36, SRB1, ABCA1 and ABCG1 mRNA expression in macrophages. Compared to that in the control group, the mRNA expression of SRB1, ABCG1, CD36 and ABCA1 in the oxLDL group was significantly increased (P<0.05), which was consistent with the findings of a previous study by our group (16). When compared to oxLDL, all of the three complexes inhibited the expression of CD36, but had no significant effect on SRB1 and ABCA1 expression (P<0.05). Only oxLDL/β2GPI and CRP/oxLDL/β2GPI significantly increased the expression of ABCG1 compared to that in the oxLDL group (P<0.05). However, there was no significant difference in the expression levels of CD36, SRB1, ABCA1 and ABCG1 mRNA among the three complexes (Fig. 2).

OxLDL and its complexes increase the secretion of inflammatory factors IL-1β, IL-6 and TNF-α by macrophages. As oxLDL is highly pro-inflammatory, the present study assessed its effect on the secretion of inflammatory factors IL-1β, IL-6 and TNF-α by RAW264.7 macrophages. Compared with the expression of IL-1β, IL-6 and TNF-α following treatment with CRP or β2GPI alone, it was significantly increased by their complexes with oxLDL. OxLDL/β2GPI inhibited the secretion of IL-1β and IL-6 induced by oxLDL (P<0.05). There were no differences in the expression levels of IL-1β and IL-6 among the oxLDL, CRP/oxLDL and CRP/oxLDL/β2GPI groups (P>0.05). Furthermore, there were no significant differences among the levels of TNF-α following treatment with oxLDL and those following treatment with any of its complexes (P>0.05) (Fig. 3).

P38/MAPK and NF-κB phosphorylation in RAW264.7 macrophages. OxLDL and its complexes significantly triggered the phosphorylation of p38/MAPK and NF-κB compared with that in the control group (P<0.05) (Fig. 4). The increase of p-p38/MAPK and p-NF-κB induced by oxLDL/β2GPI treatment was lower than that caused by oxLDL (P<0.05). The effect of CRP/oxLDL/β2GPI on activating the phosphorylation of p38/MAPK and NF-κB was identical to that of oxLDL (P>0.05). In the CRP/oxLDL group, phosphorylation of p38/MAPK and p-NF-κB was decreased compared with that in the oxLDL group, while only the difference in NF-κB phosphorylation was significant (P<0.05 vs. oxLDL).

Discussion

In a previous study by our group, animal experiments using BALB/c mice showed that exogenous CRP/oxLDL/β2GPI complexes aggravated AS in diabetic BALB/c mice by increasing lipid uptake, which was indicated to be mediated via the p38/MAPK signaling pathway (9), while the contribution of formed auto-immune antibodies to the process could not be excluded. Given that no *in vitro* study has been performed to detect the different roles of oxLDL by-product complexes on foam-cell formation and inflammatory activation without the interference of antibodies, the present study aimed to investigate the effects and the potential mechanisms of CRP, oxLDL and β2GPI as well as their complexes CRP/oxLDL, oxLDL/β2GPI and CRP/oxLDL/β2GPI on lipid accumulation and inflammatory cytokine release in RAW264.7 macrophages.

A previous study by our group has established the foam cell model *in vitro* by stimulating murine RAW264.7 macrophages with oxLDL (16). In the present study, CRP/oxLDL, oxLDL/β2GPI and CRP/oxLDL/β2GPI were able to induce macrophages to accumulate lipid and develop into foam cells; however, this effect was less marked than that of oxLDL alone, as confirmed by assessment of the cells' cholesterol content. The covalent binding of β2GPI to oxLDL reduced lipid accumulation to a significant degree, suggesting that β2GPI acts as an anti-AS agent in the process of foam cell formation and AS. With regard to the role of CRP/oxLDL, the present study showed that the levels of cholesterol in the CRP/oxLDL group were lower than those in the oxLDL group, which was consistent with the findings of other studies, which reported that macrophage binding/uptake of oxLDL was decreased when complexed with CRP (17,18). The effect of CRP/oxLDL/β2GPI on lipid accumulation in macrophages was lower than that of CRP/oxLDL, while being higher than that of oxLDL/β2GPI; however these differences were not significant. Thus, the binding of CRP or β2GPI to oxLDL was able to induce foam-cell formation, which was less marked than the effect of oxLDL on macrophage lipid uptake.

The present study further investigated the possible mechanism of lipid accumulation in macrophages. The intracellular load of oxLDL is mainly mediated by scavenger receptors, including SRBl, CD36 and lectin-like oxidized LDL receptor 1, which are expressed on the surface of macrophages, resulting in the formation of foam cells (19). ABCA1 and ABCG1 are key participants in reverse cholesterol transport, mediating cholesterol efflux directly to high-density lipoprotein

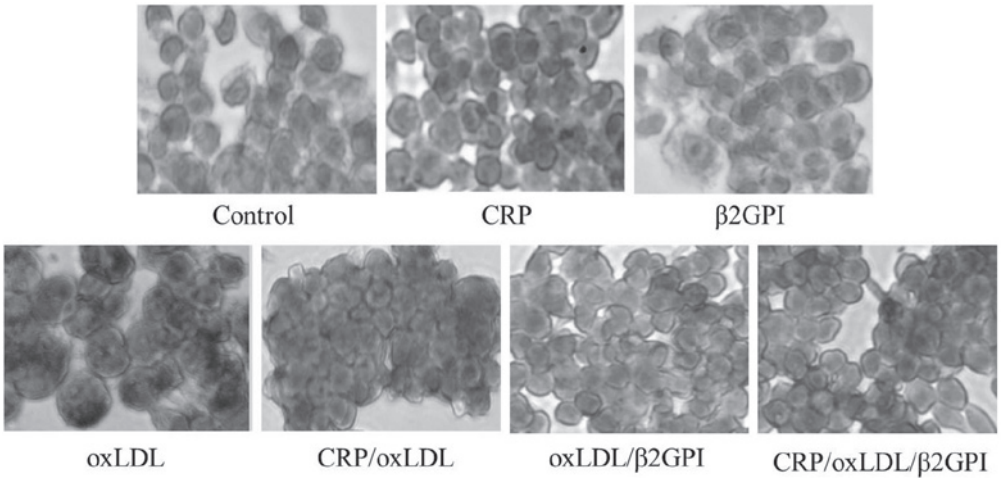

Figure 1. Lipid deposition in of RAW264.7 macrophages visualized by Oil red O staining (magnification, x320). Representative images of control, CPR, β2GPI, oxLDL, CRP/oxLDL, oxLDL/β2GPI and CRP/oxLDL/β2GPI groups. oxLDL, oxidized lipopolysaccharide; CRP, C-reactive protein; β2GPI, β2-glycoprotein I.

particles (20). The results of the present study showed that oxLDL promoted the accumulation of lipids within the cells by upregulating the expression of CD36, SRB1, ABCA1 and ABCG1 mRNA. All of the three complexes promoted CD36 mRNA expression, while their effects were less marked than that of oxLDL, which is thought to have a vital role in promoting foam-cell formation. β2GPI was indicated to have marked anti-AS properties, as expression of CD36 mRNA in the β2GPI group was as low as that in the control group, and in the oxLDL/β2GPI group, CD36 expression was significantly reduced compared with that in the oxLDL group.

Given that CRP and oxLDL are potent pro-inflammatory substances, the present study analyzed the secretion levels of IL-1β, IL-6 and TNF-α by macrophage cells in order to assess their inflammatory response induced by the complexes. IL-1β is a potent pro-inflammatory and atherogenic cytokine (21,22); in addition, macrophages are the primary IL-6 and TNF-α-producing cells. In the present study, the secretion of IL-1β and IL-6 in the oxLDL/β2GPI group was reduced compared with that in the oxLDL group. The decreased inflammatory effect of oxLDL/β2GPI implied that β2GPI binds to oxLDL, mitigating its harmful inflammatory impact and possibly exerting a protective effect in the process of foam-cell formation and AS. However, a previous *in vivo* study by our group showed that oxLDL/β2GPI increased pro-inflammatory cytokine expression under diabetic conditions when compared to oxLDL (9), which appears to contradict the results of the present *in vitro* study. The discrepancy between the *in vitro* and *in vivo* findings may be due to exogenous oxLDL/β2GPI acting as an antigen, which induced an auto-immune response and promoted the secretion of inflammatory molecules; however, this notion is required to be verified by further studies. The secretion of IL-1β, IL-6 and TNF-α stimulated by CRP/oxLDL and CRP/oxLDL/β2GPI was almost the same as that following stimulation with oxLDL; therefore, the two complexes can be regarded as pro-inflammatory agents.

Intracellular MAPK signaling cascades are important in the pathogenesis of cadiovascular diseases (23). Three MAPK families (extracellular signal-regulated kinase 1/2, p38/MAPK and c-Jun N-terminal kinase) are signaling molecules that react to extracellular stimuli and regulate immune responses (24). NF-κB activation has been linked with multiple aspects of biological functions, including inflammation, stress, cell proliferation, migration and tumour angiogenesis. A previous study by our group demonstrated that CRP/oxLDL/β2GPI increased the development and formation of AS lesions through the p38/MAPK signaling pathway in BALB/c mice (9). Furthermore, andrographolide was indicated to reduce foam cell formation by blocking the p38/MAPK and NF-κB pathways in mouse peritoneal macrophages (25). The present study explored the contribution of CRP/oxLDL/β2GPI complexes to the activation of p38/MAPK and NF-κB. The results revealed that p38/MAPK and NF-κB were involved in foam cell formation and inflammatory factor production induced by oxLDL and its complexes in RAW264.7 cells. The CRP/oxLDL complex mainly promoted the phosphorylation of p38MAPK, while CRP/oxLDL/β2GPI increased the phosphorylation of p38/MAPK as well as NF-κB *in vitro*. Hence, the induction of AS by CRP/oxLDL and CRP/oxLDL/β2GPI may be mediated via the activation of the p38MAPK and NF-κB pathways.

AS, which is a major health concern of worldwide importance, is a multifactorial process with not only lipoprotein metabolism but also inflammatory and immunological mechanisms linked to its initiation and progression (26). oxLDL is an accepted key factor in macrophage lipid accumulation and foam cell establishment, leading to the formation of atherogenic lesions and plaques (27). Circulating oxLDL has been reported to be a predictor of coronary disease in healthy middle-aged people, as well as in people with cardiovascular diseases and diabetes (28,29). While oxLDL alone is unstable in the blood circulation and is cleared rapidly, it is able to bind to β2GPI and/or CRP to form covalently stable complexes, which in turn trigger further pro-inflammatory and pro-atherogenic mechanisms.

Cu^{2+}-oxLDL-derived ligands, such as 7-ketocholesteryl-9- carboxynonanoate, mediate the interaction with β2GPI through its positively charged V domain in a time- and temperature-dependent manner (4). It has been confirmed that oxLDL/β2GPI is present in the blood circulation of patients

Table I. Effects of CRP/oxLDL/β2GPI complexes on intracellular TC content in RAW264.7 macrophages.

Group	TC concentration (μmol/mg protein)
Control	22.6±3.1
CRP	26.0±4.7[b]
β2GPI	20.3±2.5[b]
oxLDL	151.4±6.2[a]
CRP/oxLDL	124.3±4.0[a,b]
oxLDL/β2GPI	70.4±3.9[a,b]
CRP/oxLDL/β2GPI	91.2±5.8[a,b]

Values are expressed as the mean ± standard deviation of experiments performed three times in duplicate. [a]P<0.05, compared with control group; [b]P<0.05, compared with oxLDL group. TC, total cholesterol; oxLDL, oxidized lipopolysaccharide; CRP, C-reactive protein; β2GPI, β2-glycoprotein I.

with diseases, including systemic lupus erythematosus (SLE), APS, systemic sclerosis (30), diabetes mellitus (DM) (10) and chronic renal diseases (31), as well as acute coronary syndromes (32). OxLDL/β2GPI is regarded to be a pro-atherogenic auto-antigen accounting for pre-mature or accelerated arteriosclerotic cardiovascular disease in systemic auto-immune disease (33). Anti-β2GPI antibodies accelerate macrophage uptake and accumulation of oxLDL in the presence of β2GPI *in vitro* (34). OxLDL/β2GPI/anti-β2GPI was internalized by macrophages via anti-β2GPI antibody-mediated phagocytosis through Fc-γ- and Toll-like receptors, suggesting that the uptake of oxLDL/β2GPI. CRP has a crucial role in the progression and vulnerability of AS lesions (35) by inducing the production of cytokines and inflammatory mediators, including IL-1, IL-6, IL-8, TNF-α, intercellular adhesion molecule 1 and vascular cell adhesion molecule 1, which are involved in the development of AS depending on the activation of p38/MAPK, Akt and NF-κB (36,37). CRP binds to oxLDL by recognition of a phosphorylcholine moiety of oxLDL in a Ca^{2+}- and pH-dependent manner, which is a regulated mechanism of macrophages to clear CRP (7).

Unlike SLE and APS patients, in which oxLDL complexes and associated auto-antibodies are present, IgG auto-antibodies against oxLDL/β2GPI were merely observed in patients with non-autoimmune diseases, such as type 2 DM (10). CRP/oxLDL and CRP/oxLDL/ β2GPI complexes have been reported to be markedly elevated in patients with DM, rheumatoid arthritis and pyrogenic diseases, as compared with in healthy control subjects. Furthermore, the elevated complexes were reported to be associated with arterial inflammation and intima media thickness, suggesting that they may represent predictive markers with enhanced specificity for evaluating the severity of diabetic AS (11). A recent study confirmed that patients with type 2 DM exhibited significantly higher serum levels of oxLDL/β2GPI complexes than age- and gender-matched healthy control subjects. Furthermore, serum oxLDL/β2GPI levels were correlated with the severity of coronary heart disease (32).

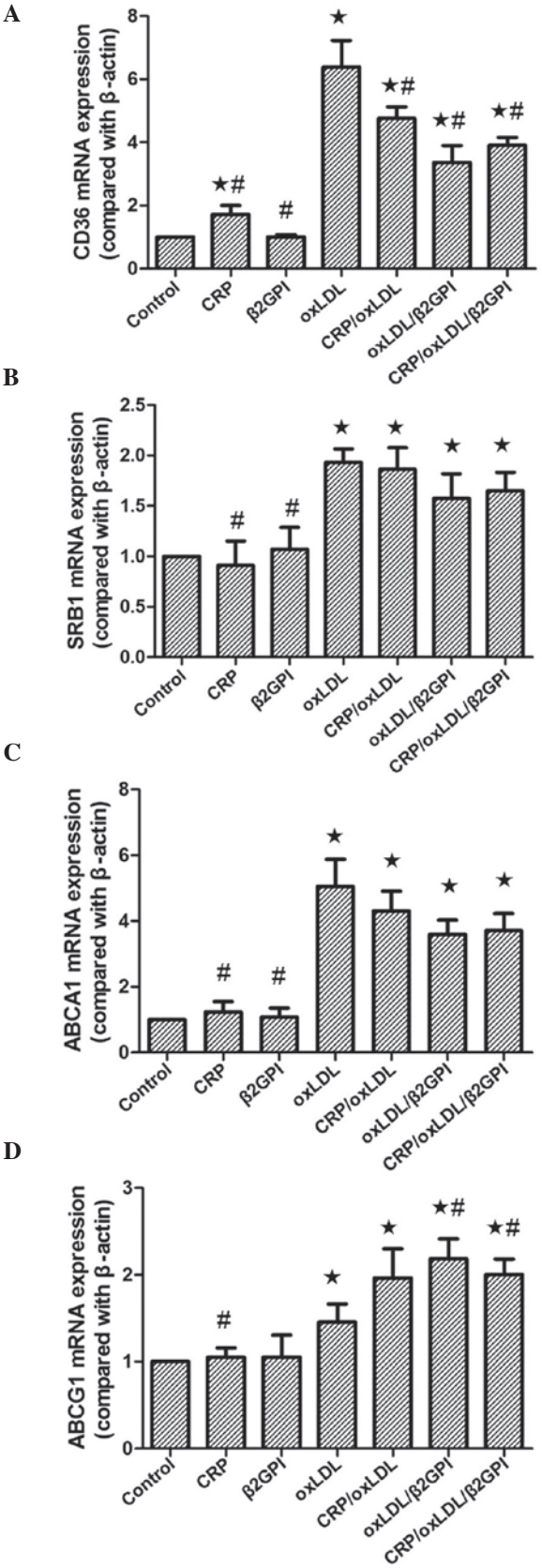

Figure 2. Effects of CRP/oxLDL/β2GPI complexes on CD36, SRB1 and ABCA1, ABCG1 mRNA expression in RAW264.7 macrophages. The mRNA expression of (A) CD36, (B) SRB1, (C) ABCA1 and (D) ABCG1 in the experimental groups was determined by reverse-transcription quantitative polymerase chain reaction analysis. Values were normalized to β-actin using the $2^{-\Delta\Delta Ct}$ method. Values are expressed as the mean ± standard deviation of experiments performed three times in duplicate. ⋆P<0.05, compared with control group; #P<0.05, compared with oxLDL group. oxLDL, oxidized lipopolysaccharide; CRP, C-reactive protein; β2GPI, β2-glycoprotein I.

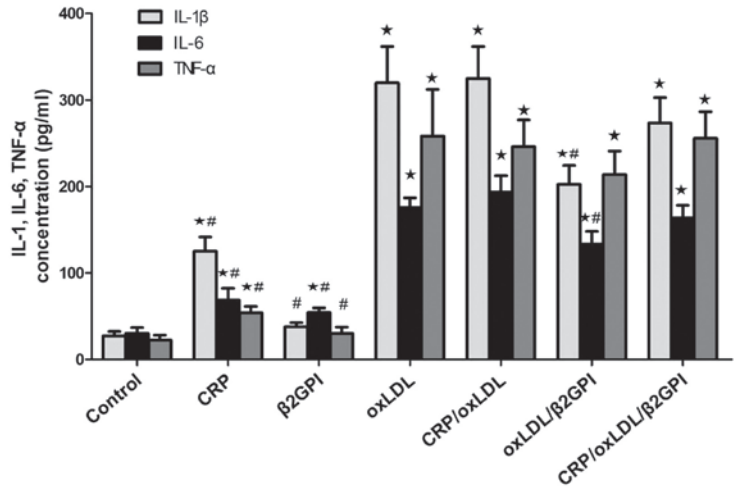

Figure 3. Effects of CRP/oxLDL/β2GPI complexes on the expression of inflammatory factors IL-1β, IL-6 and TNF-α. After treatment with CPR, β2GPI, oxLDL, CRP/oxLDL, oxLDL/β2GPI or CRP/oxLDL/β2GPI for 24 h, culture media of RAW264.7 macrophages were collected. The levels of IL-1β, IL-6 and TNF-α were measured using commercial ELISA kits. Values are expressed as the mean ± standard deviation of experiments performed three times in duplicate. ⋆P<0.05, compared with control group; #P<0.05, compared with oxLDL group. oxLDL, oxidized lipopolysaccharide; CRP, C-reactive protein; β2GPI, β2-glycoprotein I. TNF, tumor necrosis factor; IL, interleukin.

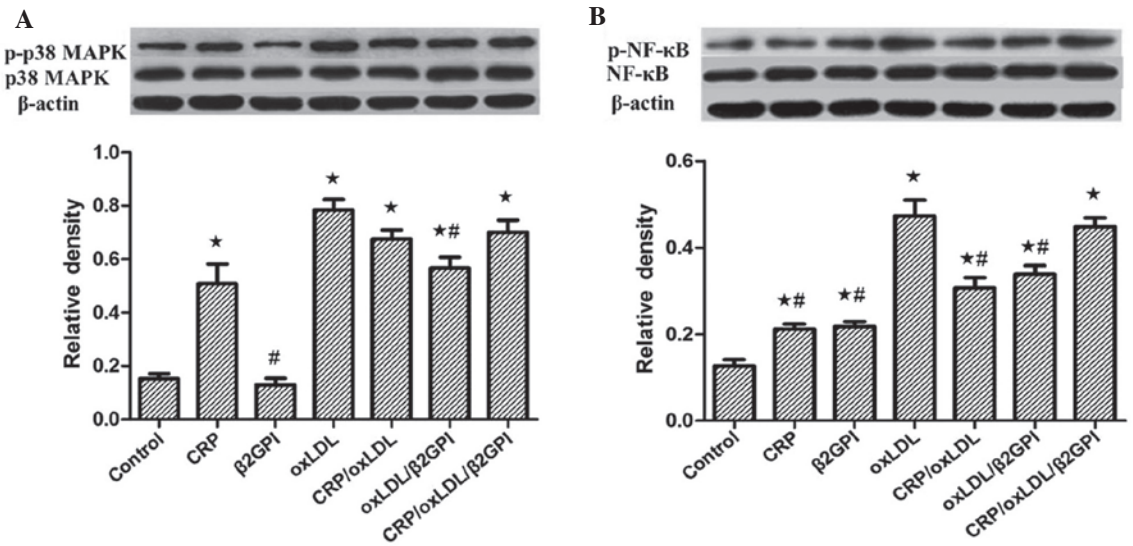

Figure 4. Effects of CRP/oxLDL/β2GPI complexes on p38/MAPK and NF-κB phosphorylation in RAW264.7 macrophages. Phosphorylated and total (A) p38/MAPK and (B) NF-κB were determined by western blot analysis. Immunoreactive bands were quantified by grey value analysis normalized to β-actin. Values are expressed as the mean ± standard deviation of experiments performed three times in duplicate. ⋆P<0.05, compared with control group; #P<0.05, compared with oxLDL group. oxLDL, oxidized lipopolysaccharide; CRP, C-reactive protein; β2GPI, β2-glycoprotein I; MAPK, mitogen-activated protein kinase; p-NF-κB, phosphorylated nuclear factor kappa B.

The present study showed that oxLDL/β2GPI was able to repress macrophage phagocytosis of lipid and decrease the expression of inflammatory factors induced by oxLDL, which is consistent with a previous study by our group, which reported that β2GPI protects macrophages from oxLDL-induced foam-cell formation and apopotosis (16). CRP/oxLDL and CRP/oxLDL/β2GPI exhibited a significant ability to induce foam-cell formation and activate inflammatory responses via p38MAPK and NF-κB phosphorylation, which was, however, decreased compared with that of oxLDL. CRP/oxLDL, oxLDL/β2GPI and CRP/oxLDL/β2GPI complexes can be distinguished from pyrogenic non-complexed CRP isoforms or unstable oxLDL and may therefore represent more specific and reliable predictive markers for AS. Thus, strategies aimed at detecting the complexes may prove to be beneficial in detecting and preventing atherothrombotic events in early stages. Among CRP/oxLDL, oxLDL/β2GPI and CRP/oxLDL/β2GPI, the complex which is most suitable as a predictive marker remains to be identified.

In conclusion, the present study indicated that CRP/oxLDL, oxLDL/β2GPI and CRP/oxLDL/β2GPI complexes induced the transformation of macrophages into foam cells by activating inflammatory responses via p38/MAPK and NF-κB signaling pathways. oxLDL/β2GPI had an anti-AS effect, but to a lesser extent than oxLDL alone. It may be possible that β2GPI has an anti-AS effect by forming a complex with oxLDL; however,

this was not demonstrated in the present study. Due to their enhanced stability in the blood compared with oxLDL, complexes of oxLDL may be suitable as predictive biomarkers of AS.

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