Advanced Oxidation Protein Products Accelerate Atherosclerosis Through Promoting Oxidative Stress and Inflammation

Shang Xi Liu, Fan Fan Hou, Zhi Jian Guo, Ryoji Nagai, Wei Ru Zhang, Zhi Qiang Liu, Zhan Mei Zhou, Mei Zhou, Di Xie, Guo Bao Wang, Xun Zhang

Objective—Increased level of plasma advanced oxidation protein products (AOPPs) has been found in patients with uremia and nonuremic subjects with coronary artery disease. This study was conducted to test the hypothesis that AOPPs play a causal role in atherosclerosis.

Methods and Results—Hypercholesterolemic (0.5% wt/wt diet) or normal rabbits received either repeated intravenous injections of AOPPs modified rabbit serum albumin (AOPPs-RSA) or unmodified RSA for 8 weeks. Compared with RSA- or vehicle-treated hypercholesterolemic rabbits, AOPPs-RSA–treated animals displayed increased atherosclerotic plaque area oxidized low-density lipoprotein (oxLDL) deposition, macrophage infiltration, and smooth muscle cell proliferation. Aortic sections from AOPPs-RSA–treated normal rabbits showed significant focal intima proliferation and mild Oil-Red-O staining lipid deposition in the affected areas, a phenomenon not observed in the RSA- or vehicle-treated controls. Plasma AOPPs levels in AOPPs-treated groups significantly increased in both hypercholesterolemic and normal rabbits compared with their relevant controls. Close correlations were found between plasma levels of AOPPs and the parameters of oxidative stress, eg, oxLDL and thiobarbituric acid reactive substances levels, or glutathione peroxidase activity. A highly significant correlation was also observed between plasma AOPPs and tumor necrosis factor (TNF)-α levels.

Conclusions—This study provides in vivo evidence for a causal relationship between chronic AOPPs accumulation and atherosclerosis. (Arterioscler Thromb Vasc Biol. 2006;26:1156-1162.)

Key Words: advanced oxidation protein products • atherosclerosis • hypercholesterolemia • inflammation • oxidative stress
or if AOPPs are actually involved in the initiation and/or progression of atherosclerosis. This study was conducted to test the hypothesis that AOPPs play a causal role for atherosclerosis.

Methods

AOPPs-RSA Preparation and Determination
AOPPs-RSA was prepared in vitro as described by Witko-Sarsat.

Briefly, rabbit serum albumin (RSA) (Sigma Aldrich, St. Louis, Mo) was exposed to HOCI (Fluke, Buchs, Switzerland) (200 mmol/L) for 30 minutes and then dialyzed overnight against PBS to remove any free HOCI. Samples were passed through a Detoxi-Gel column (Pierce, Rockford, Ill) to remove any contaminated endotoxin. Endotoxin levels in the samples were measured with the amebocyte lysate assay kit (Sigma) and were found to be below 0.025 EU/mL. AOPPs content was determined as described previously. Briefly, 200 μL of samples were placed in a 96-well plate (Corning Costar, Corning, NY) and mixed with 20 μL of acetic acid. In standard wells, 10 μL of 1.16 mol/L potassium iodide (Sigma) was added to 200 μL of chlorine-T solutions (Sigma) followed by 20 μL of acetic acid. The absorbance of the reaction mixture at 340 nm is immediately read in a microplate reader (Wallac1420, PerkinElmer, Finland). The content of AOPPs in the AOPPs-RSA was 4.3 ± 0.6 nmol/mg protein versus 0.2 ± 0.02 nmol/mg protein in unmodified RSA.

To determine whether the AOPPs preparation contains advanced glycation end products (AGEs), we measured the content of Nε-(carboxymethyl)lysine (CML), pentosidine, and glycolaldehyde (GA)-pyridine in both AOPPs-RSA and unmodified RSA as described previously. The content of CML was comparable (GA)-pyridine in both AOPPs-RSA and unmodified RSA as described previously.10–12 The content of CML was comparable between the AOPPs-RSA (44.1 ± 7.8 ng/mg protein) and unmodified RSA (40.7 ± 7.8 ng/mg protein, P > 0.05). There was no significant difference in pentosidine concentration between AOPPs-RSA (0.85 ± 0.07 nmol/mL) and unmodified RSA (0.90 ± 0.06 nmol/mL, P > 0.05). GA-pyridine was undetectable in AOPPs-RSA by both competitive and noncompetitive enzyme-linked immunosorbent assay (ELISA).

Plasma levels of AOPPs were determined as described above. To exclude the interference of turbidity of lipids on light absorption, samples were diluted 1:5 in PBS and centrifugated (10 000g, 1 hour, 4°C). The samples below the lipid layer were used for AOPPs measurement.

To quantify AOPPs in the atherosclerotic lesions, frozen tissues from aortic arch were homogenized. AOPPs levels were measured by spectrophotometry as described previously.

Animal Model
Sixty female New Zealand White rabbits (12 weeks old at the beginning of the experiments) were maintained under standardized conditions (21°C, 41% to 62% humidity) with regular day/night (12/12 hours) cycle and free access to water and diets. The animals were randomly assigned to 6 groups. Groups 1 through 3 received standard diets (K-H4 pellets, Medical Laboratory Animal Center, Gungdong); groups 4 to 6 received the same diet but supplemented with 0.5% (wt/wt) cholesterol. Animals of groups 3 and 6 received intravenous injections of 10 mg/kg AOPPs-RSA once every other day and group 2 and 5 received intravenous injections of the same dose of unmodified RSA. Control animals (groups 1 and 4) received intravenous injections of equal volume of normal saline. The dose of AOPPs and the time interval between injections were based on our preliminary experiments demonstrating that plasma AOPPs levels increased up to an extent that was similar to that found in uremic patients. At the end of 8 weeks, blood was collected into EDTA-coated tubes, and plasma was separated by centrifugation at 1000g for 10 minutes. All samples were stored at −70°C until analyzed. The tissue taken from aortic arch was stored in liquid nitrogen (LN2).

Atherosclerotic Plaque Evaluation
The entire aorta was removed, opened longitudinally, fixed in 4% buffered formaldehyde (pH 7.4), and then stained with Sudan IV to reveal sudanophilic plaque.

Images of the aorta en face were taken with a digital camera (D-100, Nikon, Japan) with a macro conversion lens (f=40 cm). The surface area of the atherosclerotic plaque was evaluated by computer-assisted morphometry as described previously. For the measurement of the thickness of the plaque, 4 to 6 sections were taken from the aortic arch, the thoracic aorta and the abdominal aorta. Sections were paraffin-embedded and stained conventionally with HE. Macroscopic analyses of the samples were performed in a blinded fashion. The mean thickness of the plaque was assessed in 4 to 6 representative sections per aortic quadrant, and the statistical mean was calculated.

Histology and Immunohistochemistry
Series sections of the aorta taken from different parts were prepared as described. Certain sections were stained with Oil-Red-O and counterstained with hematoxylin. Immunohistochemical staining were performed in other sections using monoclonal antibody HOL3 (an anti-oxidized low-density lipoprotein [oxLDL] antibody, prepared in our laboratory),

RAM11 (a macrophage-specific antibody: DAKO, Copenhagen, Denmark), and HHF35 (a muscle actin-specific antibody: DAKO, Copenhagen, Denmark) by the streptavidin-peroxidase conjugate method. The mean immunoreactive area for oxLDL was determined using an established quantitative method.

Numbers of macrophages and smooth muscle cells in the plaque were determined by counting RAM11 and HHF35 positive cells using a microscope (DM IRE2, Leica, Germany) equipped with FW4000 software. At least 5 fields were randomly chosen in each slide to get the mean number of positive cells in 0.01 mm².

Plasma Biochemical Analysis
Plasma cholesterol and triglycerides were measured by using a biochemical analyzer. Lipid peroxides were measured as thiobarbituric acid reactive substances (TBARS) by fluorometric assay.

Glutathine peroxidase (GSHPx) activity was measured as described previously. Plasma levels of oxLDL were measured by competitive ELISA using a monoclonal antibody HOL3. Plasma TNF-α was measured by a sandwich ELISA using a goat anti-rabbit TNF polyclonal antibody (BD Pharmingen) as the capture and biotinylated 23H1.1 antibody (BD Pharmingen) as the detector.

Statistical Analyses
All data are reported as mean±SD. Differences of nonplasma biochemical variables between groups were compared using 1-way ANOVA followed by LSD method if P < 0.05. The plasma biochemical variables were compared by factorial analysis. Main and interactive effect of AOPPs and hypercholesterolemia were evaluated by factorial analysis. Differences between variables of hypercholesterolemic and normocholesterolemic groups were compared by 2-sample t test. Bivariate correlations were tested using Spearman’s correlation coefficient. Statistical analyses were conducted with SPSS 12.0 for Windows. Two-tailed P < 0.05 were considered statistically significant.

Results
AOPPs Accelerate Atherosclerosis
All animals survived through the course of the experiment. Animals on the cholesterol-supplemented diet (groups 4 to 6) developed Sudan IV staining- atherosclerotic plaques. The gross plaque area was much larger in AOPPs-RSA–treated group 6 compared with vehicle-treated group 4 and RSA-treated group 5 (Figure 1). Similar results were observed for percentage of plaque surface area assessed by photoshop (Figure 2, top), as well as for average plaque thickness
The distribution of atherosclerotic plaque over entire aorta was similar in all groups of hypercholesterolemic rabbits, with the majority of lesions (41% to 54%) present in the aortic arch. The thoracic aorta contributed between 22% and 27% and the abdominal aorta contributed between 17% and 29%, respectively.

In contrast, none of the animal on normal diet developed any macroscopic plaque (Figure 1), but apparent morphological changes were found in histological sections under microscope. Most of the sections from AOPPs-RSA-treated group 3 showed focal intimal proliferation, irregular array and proliferation of smooth muscle cells and mild Oil-Red-O staining lipid deposition (Figure 1, available online at http://atvb.ahajournals.org). These phenomena did not observed in sections from vehicle-treated group 1 (Figure I) and RSA-treated group 2 (data not shown).

AOPPs Increase oxLDL Deposition, Macrophage Infiltration, and SMC Proliferation in Atherosclerotic Plaques
Immunohistochemical staining of oxLDL revealed that aortic sections from hypercholesterolemic rabbits showed positive staining of oxLDL in the plaques (Figure 3, upper panel). The mean positive staining area of oxLDL in group 6 was ~10-fold larger than that in group 4 ($P<0.0001$) or group 5 ($P<0.0001$) (Figure 3, lower panel). No significant deposition of oxLDL was found in the sections from rabbits kept on normal diet (groups 1 to 3) (data not shown).

All aortic sections from hypercholesterolemic rabbits showed significant macrophage infiltration (Figure 4, upper panel) and SMC proliferation (Figure 5, upper panel). Mean number of infiltrated macrophage increased by 1.4-fold in group 6 compared with group 4 ($P<0.05$) and increased by 1.5-fold compared with group 5 ($P<0.01$) (Figure 4, lower panel). Mean SMC number in the plaques also significantly increased in group 6 compared with group 4 and group 5 ($P<0.05$) (Figure 5, lower panel). In animals fed with normal diet, mild focal macrophage invasion and SMC proliferation could be found only in AOPPs-RSA–treated group 3 (data not shown).

AOPPs Enhances Oxidative Stress and Inflammation
As shown in the Table, plasma AOPPs levels increased by 3.0-fold in vehicle-treated hypercholesterolemic rabbits...
compared with vehicle-treated animals (6.0±1.5 nmol/mg protein) and RSA-treated animals (5.8±1.5 nmol/mg protein).

However, in hypercholesterolemic rabbits, AOPPs deposition significantly increased in AOPPs-RSA–treated rabbits (10.4±3.3 nmol/mg protein) compared with that in vehicle-treated (6.5±1.1 nmol/mg protein) and RSA-treated animals (6.2±1.4 nmol/mg protein) \( (P<0.05) \).

Plasma oxLDL, a key factor in atherogenesis, increased significantly in both hypercholesterolemic and normocholesterolemic rabbits challenged with AOPPs-RSA, the phenomenon not observed in RSA-treated animals (groups 2 and 5) compared with vehicle-treated controls (groups 1 and 4) (Table). Rabbits treated with hypercholesterolemic diet alone (group 4) also showed increased plasma levels of oxLDL compared with those fed with normal diet (group 1) \( (P<0.01) \). There was interactive effect between AOPPs and hypercholesterolemia on oxLDL levels \( (P<0.05) \). Plasma oxLDL levels significantly correlated with plasma AOPPs concentration in both normal \( (r=0.588, P<0.0001) \) and hypercholesterolemic \( (r=0.722, P<0.0001) \) animals (Figure IIA and IIB, available online at http://atvb.ahajournals.org). However, AOPPs-RSA treatment did not alter the plasma levels of triglycerides or cholesterol in either normal or hypercholesterolemic rabbits (Table).

Vehicle-treated hypercholesterolemic rabbits (group 4) showed significant increased levels of TBARS \( (P<0.001) \) and decreased activity of GSHPx compared with vehicle-treated rabbits (group 1) \( (P<0.001) \). Further elevation of plasma AOPPs concentration by intravenous injections of AOPPs-RSA significantly increased TBARS and decreased GSHPx activity in both normal (group 3) and hypercholesterolemic rabbits (group 6) as compared with their respective controls (groups 1 and 4) (Table). RSA-treatment (groups 2 and 5) did not induce any significant changes of plasma TBARS and GSHPx activity (Table). Plasma AOPPs levels positively correlated with TBARS levels in normal \( (r=0.739, P<0.0001) \) and hypercholesterolemic rabbits \( (r=0.650, P<0.0001) \) (Figure IIC and IID) and negatively correlated with GSHPx activity (normal rabbits, \( r=-0.449, P<0.01 \); hypercholesterolemic rabbits, \( r=-0.573, P<0.01 \) (Figure IIE and IIF).

Animals treated with hypercholesterolemic diet alone (group 4) showed higher plasma TNF-\( \alpha \) levels than those kept on normal diet (group 1). AOPPs-RSA treatment resulted in significant increase of plasma TNF-\( \alpha \) levels in both normal (group 3) and hypercholesterolemic rabbits (group 6) as compared with their respective controls. A close correlation was found between plasma TNF-\( \alpha \) and AOPPs levels in both normal \( (r=0.770, P<0.0001) \) and hypercholesterolemic \( (r=0.611, P<0.0001) \) rabbits (Figure IIG and IIH).

**Discussion**

In contrast to oxidized lipids, the impact of oxidized proteins in atherogenesis has not been extensively studied. Here, we found that elevation of plasma AOPPs level significantly increased atherosclerotic plaque area in hypercholesterolemic rabbits. Animals that were kept on normal diet did not develop lipid-laden plaques when
Biochemical Variables in Plasma at the End of the Experiments

<table>
<thead>
<tr>
<th>Plasma Variables</th>
<th>Normocholesterolic Rabbits (Group)</th>
<th>Hypercholesterolic Rabbits (Group)</th>
<th>Factorial ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control(1)</td>
<td>RSA(2)</td>
<td>AOPPs-RSA(3)</td>
</tr>
<tr>
<td>AOPPs, μmol/L</td>
<td>43.3±13.8</td>
<td>41.4±16.0</td>
<td>130.6±27.0*</td>
</tr>
<tr>
<td>TG, mmol/L</td>
<td>1.5±0.3</td>
<td>1.5±0.3</td>
<td>1.0±0.2</td>
</tr>
<tr>
<td>TC, mmol/L</td>
<td>0.8±0.3</td>
<td>0.8±0.3</td>
<td>0.8±0.3</td>
</tr>
<tr>
<td>OxLDL, μg/mL</td>
<td>70.8±22.9</td>
<td>72.6±14.2</td>
<td>93.9±25.7*</td>
</tr>
<tr>
<td>TBARS, μmol/L</td>
<td>4.3±1.0</td>
<td>3.2±0.8</td>
<td>6.6±1.2*</td>
</tr>
<tr>
<td>GSHPx, U/mL</td>
<td>179.9±23.8</td>
<td>182.4±37.8</td>
<td>145.9±33.7*</td>
</tr>
<tr>
<td>TNF-α, pg/mL</td>
<td>12.9±6.1</td>
<td>14.9±11.4</td>
<td>31.1±5.4*</td>
</tr>
</tbody>
</table>

Values represent mean±SD (n=10 rabbits each group).
TC indicates total cholesterol; TG, total triglycerides.
F1: F value for the main effect of AOPPs, F2: F value for the main effect of Hypercholesterolemic diet, F3: F value for the interaction of AOPPs and Hypercholesterolemia

*P<0.05 vs RSA and control at the same diet level; †P<0.05 hypercholesterolemic vs normocholesterolemic; §P<0.001; %P<0.05.

challenged by AOPPs-RSA. This finding was in line with the abundant literatures that underscore the essential role of hypercholesterolemia in disease induction. However, the early lesion of atherosclerosis did occur in normocholesterolemic animals challenged by AOPPs-RSA, as the apparent histological changes were really found in the aortic sections from these animals. AOPPs-RSA but not unmodified RSA or vehicle, promoted progression of atherosclerosis, suggesting that the atherogenetic effect was caused by AOPPs and not a property of RSA or other contaminants. These findings are in keeping with the previous report showing that atherosclerotic lesion develops in normal rabbits challenged by a glycoxidized protein referred to as AGEs.21

Exclusion of AGEs contamination in the AOPPs-RSA preparation is crucial, because exposure of proteins to HOCl in the presence of L-serine yields AGEs.14 The reaction occurs through generation of GA that formed by HOCl-serine interaction.12 However, the AOPPs-RSA used in this study was prepared by incubation of RSA with HOCl in the absence of L-serine.3 As demonstrated by Anderson et al, exposure of proteins to HOCl alone did not generate CML,25 or pentosidine (personal communication). For further confirmation, we measured the levels of CML, pentosidine, and GA-pyridine in AOPPs-RSA using specific antibodies against these epitopes. Like the previous study, we did not detect a significant level of CML, pentosidine, and GA-pyridine in our AOPPs-RSA. Thus, it seems unlikely to attribute the vascular effect of AOPPs to AGEs contamination.

It is interesting to note that AOPPs significantly increased in the homogenates of aortic tissues in AOPPs-RSA–treated animals. Though the determination of AOPPs deposition was indirect, a previous study has demonstrated that human atherosclerotic plaques were positively stained by a monoclonal antibody against HOCl-modified proteins, suggesting the presence of HOCl-modified proteins in atherosclerotic lesions.23 Hypertension is a verified risk factor for atherosclerosis. In our preliminary study,24 we observed the blood pressure in AOPPs-challenged rabbits and found that eight-week injection of AOPPs did not significantly increase the blood pressure. Taken together, these data strongly suggest that increase of plasma AOPPs, particularly in a hypercholesterolemic environment, accelerates atherosclerosis progression. To the best of our knowledge, this is the first study that provides in vivo evidence for a causal relationship between chronic AOPPs accumulation and atherosclerosis.

The mechanisms by which AOPPs accelerate atherosclerosis remain to be investigated. Although enhanced oxidative stress in uremia has been demonstrated and linked to the clinical complications such as atherosclerosis, little is known about the underlying mechanisms.25-27 In this study, we showed that plasma AOPPs levels in RSA-treated or vehicle-treated hypercholesterolemic rabbits were significantly higher than that in normal animals with the same treatment, suggesting that AOPPs spontaneously generated in hypercholesterolemia. This observation supports the previous hypothesis that hyperlipidemia may enhance the in vivo process of AOPPs formation via increase of oxidative stress.3 The occurrence of oxidative stress in hyperlipidemia has been demonstrated in the studies showing an imbalance between oxidant and antioxidant systems.28,29 Of particular interesting finding is that AOPPs loading in both normal and hypercholesterolemic rabbits were accompanied by increased oxLDL and TBARS levels (representing the levels of lipid oxidation and peroxidation) and decreased GSHPx activity (representing the capacity of antioxidant system), but not associated with alteration in lipid number or profile, suggesting that AOPPs might be not only the markers of oxidant-mediated protein damage,30 but potential inducers of oxidative stress. The close relationship between plasma AOPPs and TBARS levels or GSHPx activity and increased oxLDL deposition in aortae from animals challenged by AOPPs provided further evidence supporting the notion. Given the phenomenon that AOPPs-treated normal rabbits had significant milder atherosclerotic lesions and lower levels of plasma oxLDL compared with hyperlipidemic animals, we hypothesize that AOPPs may accelerate...
oxLDL formation through enhancing oxidative stress. The key role of oxLDL in development of atherosclerosis has been well documented. Likewise, in the in vitro studies have demonstrated that AOPPs were capable of triggering the oxidative burst of human monocyte and neutrophil. Therefore, it seems reasonable to assume that AOPPs accumulation, such as in uremia and hyperlipidemia, may constitute a new molecular basis for enhanced oxidative stress which plays central role in atherogenesis.

Much recent interest has focused on the role of an excessive inflammatory response in atherosclerosis. Although the association between atherosclerosis and inflammation has been well-documented in CRF, the initiating inflammatory factors remains largely unknown. Reactive oxygen species generated in oxidative stress has been demonstrated to be a signal for the activation of nuclear factor-kB (NF-kB), the major inflammatory transcription factor that triggers the transcription of several inflammation mediators. These inflammatory mediators can act in concert promoting atherogenesis, particularly through oxidation of LDL, leukocyte recruitment, and SMC proliferation. The potential importance of AOPPs, as the pro-oxidative factor, is that they may behave as authentic mediators of inflammation. The present study demonstrated in vivo that elevation of plasma AOPPs level resulted in excessive inflammatory response, as evidenced by increased plasma levels of TNF-α, enhanced macrophage invasion and SMC proliferation in arterial wall. Further support for the proinflammatory effects of AOPPs comes from a clinical observation showing that plasma AOPPs levels are closely correlated with the monocyte activation markers. Taken together, our findings provide new in vivo evidence suggesting that AOPPs may act as a novel class of proinflammatory mediators. Given the factor that inflammation play important role in the pathogenesis of atherosclerosis, we propose that AOPPs accumulated in the diseases such as CRF may increase oxidative stress and inflammation, and enhanced oxidative stress and inflammation can further increase AOPPs formation through stimulation of leukocytes to produce more oxidants. This positive feedback loop could amplify or maintain the oxidative stress and inflammation, and thus contribute to atherosclerosis and atheroma formation.

In summary, our findings delineate an important role for AOPPs in atherogenesis. AOPPs appear to be important components in the complex pathophysiology of oxidative stress and inflammation, and therefore should be taken as a potential target to interrupt the vicious circle of oxidative inflammation and atherogenesis.

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References


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Online supplement Figure 1

HE

Oil-Red-O

Group 1

Group 3
**Online supplement Figure 2**

**A**

$$r = 0.588, \ p < 0.0001$$

Ox-LDL, μg/mL

**B**

$$r = 0.722, \ p < 0.0001$$

**C**

$$r = 0.739, \ p < 0.0001$$

TBAES, μmol/L

**D**

$$r = 0.650, \ p < 0.0001$$

**E**

$$r = -0.449, \ p < 0.01$$

GSHPx, U/ml

**F**

$$r = -0.573, \ p < 0.01$$

**G**

$$r = 0.770, \ p < 0.0001$$

TNF-α, pg/ml

**H**

$$r = 0.611, \ p < 0.001$$