Atherosclerotic plaque disruption induced by stress and lipopolysaccharide in apolipoprotein E knockout mice

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NI, M. Wang Y, Zhang M, Zhang PF, Ding SF, Liu CX, Liu XL, Zhao YX, Zhang Y. Atherosclerotic plaque disruption induced by stress and lipopolysaccharide in apolipoprotein E knockout mice. Am J Physiol Heart Circ Physiol 296: H1598–H1606, 2009. First published March 13, 2009; doi:10.1152/ajpheart.01202.2008.—To establish an animal model with disruptions of atherosclerotic plaques, 96 male apolipoprotein E knockout (apoE<sup>−/−</sup>) mice were randomly divided into stress, lipopolysaccharide (LPS), stress + LPS, and control groups (n = 24 each). All mice were fed a high-fat diet throughout the experiment, and carotid atherosclerotic lesions were induced by placement of a constrictive perivascular collar. Four weeks after surgery, mice in the LPS and stress + LPS groups were intraperitoneally injected with LPS (1 mg/kg twice per week for 8 wk). Eight weeks after surgery, mice in the stress and stress + LPS groups were treated with intermittent physical stress (electric foot shock and noise stimulation) for 4 wk. Morphological analysis revealed a plaque disruption rate of 16.7% in control, 34.8% in LPS, 54.2% in stress, and 60.9% in stress + LPS groups. The disruption rates in stress and stress + LPS groups were both significantly higher than those of LPS groups. The disruption rates in stress and stress + LPS groups were significantly higher than those of controls (P = 0.007 and P = 0.002, respectively). Luminal thrombosis secondary to plaque disruption was observed only in the stress + LPS group. Both stress and LPS stimulation significantly decreased fibrous cap thickness and increased macrophage and lipid contents in plaques. Moreover, the combination of stress and LPS stimulation further lowered cap thickness and enhanced accumulation of macrophages and expression of inflammatory cytokines and matrix metalloproteinases. Stress activated the sympathetic nervous system, as manifested by increased blood pressure and flow velocity. Plasma fibrinogen levels were remarkably elevated in the stress and stress + LPS groups. In conclusion, stress- and LPS-costimulated apoE<sup>−/−</sup> mice provide a useful model for studies of plaque vulnerability and interventions.

atherosclerosis; inflammation; hemodynamics

Atherosclerotic plaque rupture has been identified as the major cause of acute coronary syndrome (16). A plaque with a large lipid core and a thin, weakened fibrous cap infiltrated by macrophages is prone to rupture when given extrinsic triggers (33), such as emotional stress, cold weather, and morning surge of blood pressure (BP) (34), which suggests that a sympathetic hyperactivity and hyperhemodynamic state favors the genesis of plaque rupture.

At present, no validated invasive or noninvasive methods exist to identify plaques vulnerable to rupture in patients. Efforts to identify such plaques would be greatly aided by the availability of an animal model of plaque disruption and thrombosis. An ideal animal model should combine the following features (11): the atherosclerotic process should be histologically identical to that in humans, plaque should feature the same vulnerability as its human counterpart, and plaque disruption should at least in some cases be accompanied by the formation of platelet-rich fibrin thrombi. In early cholesterol-fed rabbit models, plaque rupture was triggered by Russell viper venom and histamine (1) or mechanical strength (29). In recent years, apolipoprotein E knockout (apoE<sup>−/−</sup>) mice have been widely used for establishing an animal model of plaque rupture (7, 22, 30, 41). However, these models are still not satisfactory because of the low frequency of plaque rupture. Recently, Johnson et al. (21) observed a high frequency of plaque rupture in the brachiocephalic arteries of the model after 8 wk of a high-fat diet, with no evidence of thrombosis. von der Thüsen et al. (38) reported that the plaque rupture rate increased greatly after phenylephrine administration in p53-transformed apoE<sup>−/−</sup> mice and exhibited one case of ruptured plaque accompanied by luminal thrombosis resembling that in human lesions. Although the process triggered by phenylephrine is not physiological, it suggests that sympathetic nerve system activation may play an important role in the process of plaque rupture.

In the present study, we aimed to establish an animal model of high frequency of plaque disruption in the carotids of apoE<sup>−/−</sup> mice fed a high-fat diet and treated with stress and lipopolysaccharide (LPS) stimulation. We measured the level of stress hormones, assessed hemodynamic and coagulation status, and analyzed plaque morphology and composition and the interactive effects of stress and LPS on plaque vulnerability.

MATERIALS AND METHODS

Animal Protocol

Male apoE<sup>−/−</sup> mice (n = 96), aged 10–12 wk, were obtained from Peking University (Beijing, China). Mice were kept on a 12:12-h light-dark cycle with food and water freely available. All animals received a high-fat diet (0.25% cholesterol and 15% cocoa butter) throughout the experiment. The animal experimental protocol complied with the Animal Management Rules of the Chinese Ministry of Health (document no. 55, 2001) and was approved by the Animal Care Committee of Shandong University.

Carotid collar placement. Mice were randomly divided into four groups: stress, LPS, stress + LPS, and control groups (n = 24 each). The time line scheme of the experiment is shown in Fig. 1. Initially, all mice underwent surgery after deep anesthesia with an intraperitoneal injection of pentobarbital sodium (40 mg/kg). As described by von der Thüsen (37), carotid atherosclerotic lesions were induced by a perivascular silica collar of 3-mm length and 0.3-mm internal...
diameter placed on the left common carotid artery with an average adventitial diameter of 0.5 mm.

**Lipopolysaccharide injection.** Four weeks after surgery, mice in the LPS and stress+LPS groups were injected intraperitoneally with LPS (1 mg/kg, Sigma) twice a week for 8 wk (3, 28). Mice in the stress and control groups were injected intraperitoneally with 0.9% sodium chloride solution.

**Stress procedure.** We first performed a preliminary study to examine the effects of the two stressors, electric foot shock and noise stimulation, in isolation or in combination on hemodynamic state of mice. Our results demonstrated that systolic BP was significantly higher in mice stimulated by combined stressors (145.8 ± 5.2 mmHg) than in mice stimulated by foot shock (139.2 ± 63.3 mmHg, P = 0.034) or by noise (137.4 ± 5.4 mmHg, P = 0.009). As a result, the combinatorial stimulation was adopted as a stressing method in the present study to activate the sympathetic nervous system in mice.

Eight weeks after surgery, mice in the stress and stress+LPS groups were stimulated for 1 h by electric foot shock and noise stress every day for 4 wk. Both electric foot shock and noise stimulation were generated simultaneously by a stimulator (DI-001, Jianda Technology). The electric current was conducted by an iron bar fixed on the floor of the stimulator and was introduced every 3 min at an intensity of 0.5 mA lasting for 5 s by a previously reported method (8). Noise was produced by an alarming apparatus fixed on the top of the stimulator and was introduced every 3 min at an intensity of 110 dB lasting for 5 s by methods formerly described in mice (13, 14, 35).

The extent of sympathetic nerve system activation was evaluated by the serum level of norepinephrine, systolic and diastolic BP, heart rate (HR), maximal systolic flow velocity ($V_{\text{max}}$), and left ventricular ejection fraction (LVEF). Mice in the LPS and control groups were placed in the chamber of the stimulator without stimulation.

**Hemodynamic Assessment**

Systolic and diastolic blood pressure and heart rate in conscious mice. All mice underwent hemodynamic assessment before euthanasia. Systolic and diastolic BP and HR were measured at the end of the experiment by use of a noninvasive tail-cuff system (Softron BP-98A, Tokyo, Japan). Animals were first trained to habituate to the device to ensure accurate and reproducible measurements. Systolic BP, diastolic BP, and HR were averaged from five consecutive measurements.

**Microultrasound imaging.** Microultrasound imaging involved use of the Vevo770 high-resolution microultrasound imaging system (Visualsonics, Toronto, ON, Canada) to measure the $V_{\text{max}}$ of the left carotid artery and LVEF for assessing systemic hemodynamic status. Animals were anesthetized with 2% isoflurane gas, and HR in the four groups of mice dropped similarly to ~350 beats/min after anesthesia. The temperature of animals was monitored by use of a rectal probe and regulated with a heating pad. Electrocardiographic monitoring was performed with a lead II limb lead. A 50-MHz mechanical transducer, with a 4.5-mm focus, was oriented at <60° to the left carotid arterial flow to record $V_{\text{max}}$ with pulse-wave Doppler technique. Subsequently, a 40-MHz mechanical transducer was used to measure the left ventricular end-systolic and end-diastolic diameters from the parasternal long-axis view. Off-line measurements involved use of an image analysis system. LVEF was calculated according to formulas published previously (42).

**Serum Norepinephrine and Biological Measurements**

Immediately after the last stress stimulation, mice were anesthetized by intraperitoneal pentobarbital injection, and blood samples were taken from retroorbital bleeding. Serum was separated by centrifugation at 4°C, and the serum level of norepinephrine was detected by enzyme-linked immunosorbent assay (Rapibio Lab) within 30 min. Total cholesterol and triglyceride levels were measured by enzymatic methods. High-density lipoprotein (HDL) cholesterol was determined after precipitation of apolipoprotein B by enzymatic methods. Low-density lipoprotein (LDL) cholesterol was calculated according to Fredewald’s formula. Plasma was collected for measurement of fibrinogen levels by the physiological coagulation method.

**Histological and Morphological Analyses**

Mice were anesthetized by intraperitoneal pentobarbital injection. Mice were perfused through the left ventricle with phosphate-buffered saline, followed by 4% paraformaldehyde under physiological pressure. The left common carotid artery was dissected and fixed in 4% paraformaldehyde overnight and then embedded in OCT compound. Serial cryosections were cut at 6-μm thickness every 50 μm along the carotid artery specimens, and ~100 sections per vessel were obtained. Sections were stained with hematoxylin and eosin. Collagen was visualized by Masson’s trichrome and sirius red staining. Elastin was visualized by Verhoeff staining. Lipid core was identified by oil red O staining. Perl’s staining was applied to display plaque hemorrhage, with positive-staining areas indicating iron deposition that was released from erythrocytes phagocytosed by macrophages. The remaining sections were used for immunohistochemical analysis with the following antibodies: anti-mouse monocyte/macrophage monoclonal antibodies (MOMA-2, 1:25, Serotec), anti-α-smooth muscle (SM) actin monoclonal antibodies (1:1,000, Sigma), anti-human interleukin-6 (IL-6) (6-polyclonal antibodies (1:400, Abcam), anti-human tumor necrosis factor-α (TNF-α) monoclonal antibodies (1:50, Abcam), anti-mouse monocyte chemoattractant protein-1 (MCP-1) antibodies (1:20, R&D Systems), anti-human matrix metalloproteinase (MMP)-2 monoclonal antibodies (1:200, Abcam), anti-mouse MMP-9 polyclonal antibodies (1:500, Abcam), and anti- mouse MMP-12 antibodies (1:500, RayBiotech). After incubation with the appropriate horseradish peroxidase-conjugated secondary antibodies (1:100, Zhongshan Biological Technology), the sections were incubated with 3,3’-diaminobenzidine and counterstained with hematoxylin. Negative control was added with the normal IgG without the primary antibodies.

The point of maximal stenosis of each artery was determined by analysis of sections at 50-μm intervals. The site of maximal plaque showed the highest optical density, and the cap-to-core area ratio was then derived. Lipid, collagen, and immunostaining positive areas were quantified by computerized image analysis of sections at 50-μm intervals. The cap-to-core area ratio was then derived. Lipid, collagen, and immunostaining positive areas were quantified by computerized image analysis of sections at 50-μm intervals. The cap-to-core area ratio was then derived. Lipid, collage,
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20). For calculating plaque disruption rate, a given animal was counted only once no matter how many sections from one specimen demonstrated evidence of plaque disruption.

Statistical Analysis

Data are expressed as means ± SD. The statistical design was a 2 × 2 factorial, with stress and LPS as two factors. Factorial ANOVA was conducted to analyze the main effects and interactive effects of stress and LPS. Multiple comparisons were analyzed by least significant difference (LSD) post hoc tests. Frequency data analysis involved use of Pearson χ². Linear regression analysis was used to test correlations between hemodynamic parameters and histological evidence of plaque rupture. A level of P < 0.05 was considered statistically significant. All analyses involved the use of SPSS 13.0 (SPSS, Chicago, IL).

RESULTS

Body Weight

Body weight did not differ among the four groups of mice. One mouse in the LPS group and one mouse in the stress group died after the first LPS injection (Table 1). The feet of each animal were checked after electric stimulation, and no apparent skin injury was found.

Hemodynamic Status

Systolic and diastolic blood pressure and heart rate. Stress had significant effects on BP and HR (both P < 0.001). Both systolic BP and diastolic BP measured in awake, unanesthetized mice were significantly higher in the stress and LPS groups than in the LPS and control groups (Table 1). Also, HR measured in unanesthetized mice was significantly higher in the stress and stress + LPS groups than in the LPS and control groups (Table 1).

Muscoultrasound imaging. Stress exhibited a prominent effect on the values of Vmax and LVEF (both P < 0.001), which were significantly increased in the stress and stress + LPS groups compared with the LPS and control groups (Table 1).

Table 1. Basic characteristics and biochemical parameters in four groups of mice

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control (n = 24)</th>
<th>LPS (n = 23)</th>
<th>Stress (n = 24)</th>
<th>Stress + LPS (n = 23)</th>
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<tr>
<td>Body weight, g</td>
<td>26.9 ± 1.2</td>
<td>26.4 ± 1.1</td>
<td>26.7 ± 1.4</td>
<td>26.9 ± 1.2</td>
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<td>Systolic BP, mmHg</td>
<td>105.3 ± 5</td>
<td>107.7 ± 2.5</td>
<td>143.1 ± 6.3</td>
<td>143.7 ± 12.1</td>
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<tr>
<td>Diastolic BP, mmHg</td>
<td>77.1 ± 7.3</td>
<td>80.1 ± 6.3</td>
<td>100.9 ± 5.3</td>
<td>101.8 ± 8.2</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>509.5 ± 74.7</td>
<td>580.2 ± 89.7</td>
<td>662.7 ± 57.1</td>
<td>654.8 ± 61.6</td>
</tr>
<tr>
<td>LVEF, %</td>
<td>61.5 ± 3.8</td>
<td>61.9 ± 5.8</td>
<td>72.1 ± 5.5</td>
<td>74.4 ± 4.9</td>
</tr>
<tr>
<td>Vmax, mm/s</td>
<td>640.108</td>
<td>689 ± 74</td>
<td>1335 ± 205</td>
<td>1430 ± 301</td>
</tr>
<tr>
<td>Norepinephrine, ng/ml</td>
<td>83.9 ± 5.8</td>
<td>73.9 ± 7.0</td>
<td>464 ± 36.4</td>
<td>450 ± 37.9</td>
</tr>
<tr>
<td>Total cholesterol, mmol/l</td>
<td>15.17 ± 3.5</td>
<td>17.50 ± 4.29</td>
<td>18.38 ± 4.55</td>
<td>16.24 ± 4.02</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/l</td>
<td>5.20 ± 1.22</td>
<td>5.19 ± 1.35</td>
<td>5.10 ± 1.25</td>
<td>5.50 ± 1.47</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/l</td>
<td>1.48 ± 0.27</td>
<td>1.53 ± 0.35</td>
<td>1.44 ± 0.27</td>
<td>1.51 ± 0.24</td>
</tr>
<tr>
<td>Triglyceride, mmol/l</td>
<td>0.42 ± 0.14</td>
<td>0.44 ± 0.09</td>
<td>0.49 ± 0.14</td>
<td>0.53 ± 0.17</td>
</tr>
<tr>
<td>Fibrinogen, g/l</td>
<td>2.12 ± 0.21</td>
<td>2.15 ± 0.33</td>
<td>2.49 ± 0.21</td>
<td>3.09 ± 0.38</td>
</tr>
</tbody>
</table>

Values are means ± SD for n animals. LPS, lipopolysaccharide; BP, blood pressure; HR, heart rate; LVEF, left ventricular ejection fraction; Vmax, maximal systolic flow velocity; LDL, low-density lipoprotein; HDL, high-density lipoprotein. *P < 0.001 vs. control group, **P < 0.001 vs. LPS group, *P < 0.001 vs. stress group, **P = 0.007 vs. LPS group, *P = 0.049, interaction between stress and LPS (factorial ANOVA).

Atherosclerotic plaques were well developed in the left carotid arteries after 12-wk collar placement. In contrast, no atherosclerotic plaques were found in the right carotid arteries without collar placement during the same experimental period of time. Characteristics of plaque phenotype are displayed in Table 2. Morphological analysis revealed features of plaque disruption in 4 of 24 mice (16.7%) in the control group, 8 of 23 (34.8%) in the LPS group, 13 of 24 (54.2%) in the stress group, and 14 of 23 (60.9%) in the stress + LPS group. The plaque disruption rate in the stress and stress + LPS groups was significantly higher than that in the control group. Although more plaque disruption was observed in the LPS group than in the control group, the difference was not statistically significant. Buried fibrous cap and intraplaque hemorrhage were observed in all groups of mice except for the control group. Consecutive analysis of the serial sections in each specimen revealed that there was always a discontinuity of the buried cap at the end of the healed rupture lesion. Luminal thrombosis associated with cap disruption was found only in the stress + LPS group.

The plaque area did not differ among the control group (45,000 ± 13,000 μm²), the LPS group (48,000 ± 12,000 μm²), the stress group (52,000 ± 16,000 μm²), and the stress + LPS group (54,000 ± 15,000 μm²). However, the fibrous cap thickness and the cap-to-core area ratio showed a significant difference among these groups. The mean cap thickness in the stress + LPS group (6.04 ± 1.78 μm) was less than that of the control group (14.63 ± 2.80 μm, P < 0.001), the LPS group (9.60 ± 2.61 μm, P = 0.001), and the stress group (8.59 ± 2.45 μm, P = 0.002; Fig. 3A). Stress or LPS stimulation alone had a significant effect (both P < 0.001),
while the two stimulations in combination had an interactive effect on attenuating the fibrous cap thickness ($P = 0.015$). Similarly, the cap-to-core area ratio in the stress + LPS group (6.11 ± 1.45%) was significantly less than that of the control group (15.49 ± 1.46%, $P < 0.001$), the stress group (8.84 ± 1.43%, $P < 0.001$), and the LPS group (9.50 ± 1.57%, $P < 0.001$; Fig. 3B). Stress or LPS stimulation alone decreased the cap-to-core area ratio (both $P < 0.001$), and the two stimulations in combination had a synergistic effect on this ratio ($P = 0.001$).

**Effects of Stress and LPS on Plaque Composition**

The composition of plaques, including lipids, collagen, macrophages, and smooth muscle cells (SMCs) were demonstrated by oil red O staining, sirius red staining, and MOMA-2 and α-SM actin immunohistochemical staining, respectively (Fig. 4). Both LPS and stress had significant effects on decreasing the collagen content and increasing the lipid and macrophage contents in plaques (all $P < 0.001$). Furthermore, the combination of LPS and stress had a significant synergistic effect on plaque composition of collagen ($P = 0.004$), lipids ($P = 0.002$), and macrophages ($P < 0.001$). Plaques in the stress + LPS mice showed a significantly lower collagen content ($P < 0.001$ vs. control group, $P < 0.001$ vs. LPS group, and $P = 0.001$ vs. stress group), a higher lipid content ($P < 0.001$ vs. control group, $P = 0.007$ vs. LPS group, and $P = 0.001$ vs. stress group), and a higher macrophage content ($P < 0.001$ vs. control group, $P = 0.001$ vs. LPS group, and $P = 0.031$ vs. stress group) than plaques in the other three groups (Fig. 3, C–E). In contrast, there was no significant difference in SMC content among the four groups of mice (Fig. 3F).

The effects of stress and LPS on the expression level of inflammatory cytokines (IL-6, TNF-α, and MCP-1) and MMPs (MMP-2, MMP-9, and MMP-12) within the lesions were examined by immunohistochemistry (Fig. 5). For most of these inflammatory mediators, LPS or stress stimulation alone had no effects on expression level. However, these effects became significant when the two stimulations were combined, because plaques of the stress + LPS mice showed higher expression levels of inflammatory cytokines than those of the mice in the control group (Fig. 6).

**Correlations Between Hemodynamic Parameters and Histological Evidence of Plaque Disruption**

Significant positive correlations were found between histological evidence of plaque disruption and systolic BP, diastolic BP, $V_{\text{max}}$ of the left carotid artery, and LVEF by linear regression analyses (Table 2).

**DISCUSSION**

In the present study, we established an animal model with a vulnerable plaque phenotype by simulating some risk factors that can cause plaque rupture in humans, including high-fat diet, inflammation, and stress stimulation. Although stress and LPS stimulation had similar effects on plaque vulnerability, such as decreased fibrous cap thickness and increased plaque...
lipid and macrophage contents, the plaque disruption rate tended to be higher in the stress group than in the LPS group, which supports the idea that a hyperhemodynamic state plays an important role in triggering vulnerable plaques to rupture. We found that the combination of stress and LPS stimulation induced the highest plaque disruption rate among the four groups of mice, especially with luminal thrombosis. The intensified inflammation evidenced by high levels of inflammatory mediators and the hypercoagulative state indicated by high levels of plasma fibrinogen may both contribute to this phenomenon.

Although it has been recognized that both plaque vulnerability (intrinsic features) and rupture triggers (extrinsic forces) are essential for plaque disruption, an ideal animal model mimicking spontaneous plaque disruption in humans is still lacking. von der Thusen et al. (38) found that carotid plaques became vulnerable after p53 gene was overexpressed in apoE/H11002/H11002 mice, and the mechanisms may involve excessive apoptosis of vascular SMCs and a marked decrease in the cellular and extracellular contents of the fibrous caps. They also found that plaque rupture rate increased significantly after phenylephrine triggering. However, this model does not fully resemble human plaque rupture and intraluminal thrombosis, because the triggering process is by no means pathophysiologically. Spontaneous plaque disruption has been reported in the aortic or brachiocephalic arteries of apoE/H11002/H11002 and LDL receptor knockout (LDLR−/−) mice (7, 21, 22, 30, 41). However, the frequency of plaque disruption is not high enough to allow for interventional study.

In apoE−/− mice, the atherosclerotic lesions are partial to the large arteries, primarily the aortic root, the lesser curvature of the outflow track, and the descending aorta (25). Rosenfeld and Schwartz (31) believe that the most reproducible site for studies of advanced lesions in mice is the carotid artery, which is a better model for human coronary artery disease than, for instance, the ascending aorta of the apoE/H11002/H11002 mouse or for that matter the typical lesions that rarely progress to clinically significant status as seen in rabbits, monkeys, and pigs. As for the coronary arteries of older apoE−/− mice, the major lesions were found to locate in the valve sinus, including the origins of the coronary arteries. However, the first segment and first branch of all the major coronary arteries, the usual sites of disease in humans, were protected from disease (18). In the present study, the common carotid artery of apoE/H11002/H11002 mice was chosen as an ideal arterial site for plaque characterization for the following reasons. First, carotid plaques induced by a constrictive collar develop much faster than those of other arteries such as the aorta and the coronary arteries, which showed extensive atherosclerosis only in rather old apoE−/− mice (aged 9–20 mo) (7). This feature is an important advantage for many interventional studies. Second, unlike the aorta, brachiocephalic artery, and coronary arteries, the common

![Fig. 3. Comparison of atherosclerotic plaque parameters among groups. Significant differences in cap thickness (A), cap-to-core area ratio (B), collagen content (C), lipid deposition (D), and macrophage content (E) among groups are shown. There was no statistical difference in the content of smooth muscle cells (SMCs) among groups [α-smooth muscle (SM) actin; F]. Stress+LPS-treated plaques showed the lowest levels of cap thickness, cap-to-core ratio, and collagen and the highest levels of lipids and macrophages among the groups. Values are means ± SD. *P < 0.05 vs. control; †P < 0.01 vs. LPS; ‡P < 0.05 vs. stress; §P < 0.05, interaction between stress and LPS, factorial ANOVA.](http://ajpheart.physiology.org/ftp/ajpheart/296/2/1602/fig3.jpg)
carotid artery of apoE<sup>−/−</sup> mice is easily accessible and can be repeatedly exposed as needed for gene or pharmacological interventions (12, 38, 43). Third, the morphological evolutions of the carotid plaques of apoE<sup>−/−</sup> mice can be noninvasively evaluated by imaging technologies. Recently, using a microultrasound imaging system, we found (26) that the intima-medial thickness and the maximal flow velocity of the carotid artery were independent predictors of plaque rupture in stress-LPS-treated apoE<sup>−/−</sup> mice.

Stress as a more physiological stimulus than pharmacological or mechanical triggers was found to have significant effects on plaque vulnerability in the present study. Epidemiologic evidence supports the idea that stress, which can activate the sympathetic nervous system, is a risk factor for acute cardiovascular events (10, 40). Hemdahl et al. (17) reported that both stress and hypoxic stimulation could trigger myocardial infarction in apoE<sup>−/−</sup>/LDLR<sup>−/−</sup> mice. In the present study, stress acted as an ideal physiological trigger instead of pharmacological triggers to induce plaque disruption. Previous studies have confirmed that both electric foot shock and noise stimulation are effective stressors for mice (8, 13, 23, 24, 27, 35, 44). Besides the hemodynamic effects, foot shock stress also affects inflammatory (8) and immunologic (24) function.

Noise stress has been found to induce neurological dysfunction, promote epinephrine release (32), increase BP (2), and enhance free oxygen radical production (13, 35). In this study we first combined electric foot shock and noise stimulation together in apoE<sup>−/−</sup> mice in order to produce a more effective stressor, and our results demonstrated that stress-treated mice indeed had high levels of serum norepinephrine and hyper-hemodynamic states manifested by elevated systolic BP, diastolic BP, HR, LVEF, and V<sub>max</sub>. Surging hemodynamic forces caused by stress would contribute to flow turbulence and increased shear stress in the plaque site and precipitate plaque disruption. However, to ensure high experimental safety, we used an intermittent rather than continuous stimulation protocol, and the total duration of electric and noise stimulation (100 s/day) in this study was much shorter than that in previous stress studies (8). Consequently, physical examinations revealed no injury in our apoE<sup>−/−</sup> mice after the experiment.

Enhanced inflammation is a characteristic of vulnerable plaques in stress- and stress+LPS-treated mice. Inflammation plays an important role in the process of plaque rupture and subsequent thrombosis (9). Previous studies demonstrated that both LPS and stress could induce a chronic inflammatory process (5, 19, 36). Inflammatory events induced by stress may

Fig. 4. Atherosclerotic plaque composition on histology. Combination of stress and LPS significantly increased levels of lipids (A–D, oil red O staining) and macrophages (I–L, MOMA-2 immunostaining) and decreased levels of collagen (E–H, sirius red staining) in plaques. No difference in SMCs was found among groups (M–P, α-SM actin immunostaining).
account for 40% of atherosclerotic events in patients with no other known risk factors (4). Increased inflammation is most likely the reason for the adverse events in stress and LPS mice, as demonstrated by the enrichment of cytokines, chemokines, and macrophages in plaques.

A hypercoagulative reaction of blood is another characteristic of the stress and stress + LPS-treated mouse model. Previous studies showed that stress is related to a hypercoagulative state as reflected by increased level of procoagulant molecules (i.e., fibrinogen or coagulation factor VII) and by reduced fibrinolytic capacity (39). As well, increased platelet adhesion and aggregation induced by stress play a crucial role in thrombosis secondary to plaque rupture (6).

Our study contains several limitations. First, although mice in the stress + LPS group showed more features of vulnerable plaques, the incidence of plaque disruption did not show significant difference among the three intervention groups. This is probably because the number of animals in each group was still too small to predict the difference in plaque disruption between the stress + LPS group and groups exposed to stress or LPS alone. Second, although a hyperdynamic state was successfully induced in our animal model, the effects of antihypertensive therapy in preventing plaque disruption were not examined, and thus further studies are required to look at this relation. Third, the experimental procedures for inducing plaque rupture are still artificial, and further studies are required to develop an animal model with a high frequency of spontaneous plaque rupture.

In conclusion, carotid atherosclerotic plaques in stress + LPS-treated apoE−/− mice share many characteristics of the vulnerable plaques of humans. The distinctive pathophysiological mechanisms by which stress combined with LPS treatment promotes plaque disruption include augmented hemodynamic reactivity, blood coagulation, and inflammatory processes. This model may provide not only a tool for examining the mechanisms of plaque vulnerability but also an accessible site for evaluating the therapeutic effects of local transgenes or pharmacological interventions.

Table 2. Correlations between hemodynamic parameters and plaque disruption

<table>
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<tr>
<th>Hemodynamic Parameters</th>
<th>r</th>
<th>P</th>
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<tbody>
<tr>
<td>Systolic BP</td>
<td>0.53</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diastolic BP</td>
<td>0.43</td>
<td>0.006</td>
</tr>
<tr>
<td>V_{max}</td>
<td>0.46</td>
<td>0.015</td>
</tr>
<tr>
<td>LVEF</td>
<td>0.47</td>
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