Activation of the TLR4 signaling pathway and abnormal cholesterol efflux lead to emphysema in ApoE-deficient mice

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Submitted 18 December 2010; accepted in final form 21 March 2012

Am J Physiol Lung Cell Mol Physiol 302: L1200–L1208, 2012. First published March 23, 2012; doi:10.1152/ajplung.00454.2010.—Smokers with airflow obstruction have an increased risk of atherosclerosis, but the relationship between the pathogenesis of these diseases is not well understood. To determine whether hypercholesterolemia alters lung inflammation and emphysema formation, we examined the lung phenotype of two hypercholesterolemic murine models of atherosclerosis at baseline and on a high-fat diet. Airspace enlargement developed in the lungs of apolipoprotein E-deficient (ApoE−/−) mice exposed to a Western-type diet for 10 wk. An elevated number of macrophages and lymphocytes accompanied an increase in matrix metalloproteinase-9 (MMP-9) activity and MMP-12 expression was observed in the lungs of ApoE−/− mice on a Western-type diet. In contrast, low-density lipoprotein receptor-deficient (LDLr−/−) mice did not exhibit lung destruction or inflammatory changes. Most importantly, we revealed augmented expression of the downstream targets of the Toll-like receptor (TLR) pathway, interleukin-1 receptor-associated kinase 1, and granulocyte colony-stimulating factor, in the lungs of ApoE−/− mice fed with a Western-type diet. In addition, we demonstrated overexpression of MMP-9 in ApoE−/− macrophages treated with TLR4 ligand, augmented with the addition of oxidized LDL, suggesting that emphysema in these mice results from the activation of the TLR pathway secondary to known abnormal cholesterol efflux. Our findings indicate that, in ApoE−/− mice fed with a Western-type diet, activation of the TLR4 pathway secondary to known abnormal cholesterol efflux, suggesting a link between atherosclerosis and obstructive lung disease (32).

One of the major hallmarks of emphysema and atherosclerosis is inflammation originating from the infiltration of macrophages and lymphocytes into the airway and vessel wall, respectively. Atherosclerotic lesions have increased numbers of lipid-laden macrophages (23). Likewise, smokers with airflow limitations exhibit an increase in the number of macrophages and T-lymphocytes in the lung, the presence of which correlate with the development and progression of emphysema (34, 60). Animal models of atherosclerosis and emphysema demonstrate similar inflammatory profiles (50, 54). Therefore, we examined the lungs of two murine models of atherosclerosis to assess the potential consequences of hyperlipidemia on lung structure.

The most widely used murine models for atherosclerosis are apolipoprotein E (ApoE)-deficient mice and LDL receptor-deficient mice, which both develop hypercholesterolemia (33, 58). Under normal conditions, ApoE accepts cholesterol from cells and transports it back to the liver, where it can be excreted. The loss of ApoE and the LDL receptor result in hypercholesterolemic states as a result of impaired lipoprotein production and metabolism (33, 58). One of the critical roles of ApoE is to promote cholesterol efflux from macrophages (8, 37). Deficiency of endogenous ApoE expression leads to the deleterious effects of cholesterol-overloaded macrophages, which are also known as foam cells (42). ApoE promotes macrophage cholesterol efflux through the ABCA-1 and ABCG-1 cell surface transporters, which facilitate the efflux of phospholipids and cholesterol onto lipid-poor apolipoproteins, thereby initiating the formation of high-density lipoprotein (HDL) particles (53). Accumulation of oxidized LDL in the macrophages of the atherosclerotic plaque stimulates the secretion of various cytokines and proteases, which leads to degradation of the extracellular matrix (45). In contrast to ApoE, the LDL receptor mediates the binding and endocytosis of excess circulating LDL cholesterol to liver cells, where the cholesterol is catabolized and ultimately secreted in the feces via the biliary pathway (17). The goal of the present study was to examine the lung phenotype in ApoE−/− and LDLr−/− to determine whether the lung inflammation or parenchymal structure is altered in these mouse models of atherosclerosis.

* M. Goldklang and P. Golovatch contributed equally to this work.

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MATERIALS AND METHODS

Animal experiments. Two murine models of atherosclerosis were used to study the effect of hypercholesterolemia on the lung structure. The control group consisted of female C57BL6/J mice obtained from Jackson Laboratories fed chow diet (n = 5) or Western-type diet (n = 5) for 10 wk. The first experimental group included 8-wk-old female Apoe−/− mice (n = 6) subjected to a Western diet for 10 wk, compared with Apoe−/− mice on a chow diet (n = 5). In the second experimental group of animals, the Ldlr−/− model of atherosclerosis was used to observe the effect of the diet on the lungs. Ldlr−/− mice were exposed to a Western diet (n = 6) or chow (n = 6) for 10 wk. Female mice were selected on the basis of their increased susceptibility to the atherosclerotic plaque formation (56). Mice were fed an atherogenic high-fat Western-type diet (20% protein, 50% carbohydrate, 21% fat, 0.21% cholesterol; D12079B; Research Diets, New Brunswick, NJ). The animal experiments were repeated at least two times for each subgroup. Animals were housed at Columbia University Medical Center according to animal welfare guidelines. Food and drinking water were provided ad libitum. The Columbia University Institutional Animal Care and Use Committee approved all animal studies.

Histology and immunohistochemistry. After exposure to the Western or chow diet, mice were anesthetized with isoflurane and killed by carbon dioxide inhalation. The trachea was cannulated with a 16-gauge argon catheter secured with a silk suture. The lungs were washed first with PBS (1 ml) to collect bronchoalveolar lavage (BAL) fluid, and then pressure was perfused with 10% formalin to 25 cm H2O for 20 min. Tissues were stored in formalin for at least 24 h before paraffin embedding and sectioning (4 μm). Sections were stained with hematoxylin and eosin (H&E) for histological analysis including morphometry and quantification of inflammatory cells. Morphometric analysis of the H&E-stained lungs was performed as previously described (20, 46). Morphometric assessment was conducted to determine the average distance between alveolar walls (mean linear intercept), the fractional volume of parenchyma tissue per lung, the alveolar surface area per unit volume, and macrophage and lymphocyte counts. Forty histological fields (×40 magnification) were analyzed from at least four separate, randomly chosen sections from each mouse to calculate morphometric parameters. We adhered to a strict protocol for blinding and randomization (30), including a random number generator to select slides for analysis, as well as use of the OptiScan II motorized stage (Prior Scientific, Rockland, MA) controlled by Image-Pro Plus 7.0 software (Media Cybernetics, Bethesda, MD).

Serum total cholesterol measurements. Utilizing the Wako Cholesterol E kit (Wako Pure Chemical Industries, Osaka, Japan), total serum cholesterol measurements were performed. Blood was obtained from mice at the time of death via cardiac puncture, and serum was separated and frozen for later analysis. Due to high cholesterol levels, serum cholesterol measurements were performed. Blood was obtained from mice at the time of death via cardiac puncture, and serum was separated and frozen for later analysis. Due to high cholesterol levels, serum cholesterol was repeated on three subsequent days to ensure accuracy.

Cell culture. Peritoneal macrophages were obtained from Apoe−/− and Apoe+/+ mice following intraperitoneal thioglycollate injection. The macrophages were cultured in DMEM containing 5% PBS for 24 h and subsequently treated with ligands for Toll-like receptor 2 (TLR2) (peptidoglycan, 2 μg/ml), TLR3 (polyinosine-polyctydylid acid, 2.5 μg/ml), and TLR4 (lipid A, the active component of lipopolysaccharide, 100 ng/ml) (InvivoGen, San Diego, CA). Macrophages were also treated with both TLR4 ligand and oxidized LDL (100 μg/ml). All cell culture experiments were repeated at least three times on subsequent days to ensure accuracy.

Western blotting. Freshly dissected lungs of Apoe−/− and Ldlr−/− mice (10 mg) were homogenized in 1 ml of protein lysis buffer (PBS containing Triton X-100 0.1%, and centrifuged (14,000 g for 10 min). Fifty micrograms of the lung lysates of each group were subjected to Western Blot analysis. Rabbit polyclonal antibodies against phospho- (ph-) ERK, total ERK, ph-JNK (Cell Signaling, Beverly, MA), and interleukin-1 receptor activated kinase (IRAK)-1 (Santa Cruz Biotechnology, Santa Cruz, CA) were used, following the manufacturer’s instructions. A goat polyclonal antibody against β-actin (Santa Cruz) was utilized to assess for equal protein loading. Western blots were performed twice for accuracy.

Zymography. Gelatin zymography was performed using bronchoalveolar lavage fluid to detect proteases having gelatinolytic activity, including matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9), as previously reported (26). Zymography was performed twice to ensure accuracy of the results.

Statistical analysis. Statistics were performed using Prism 5.0d software. Linear regression models were used to examine the relationship between total serum cholesterol and macrophage count in Apoe−/− and Ldlr−/− mice. Separate slopes and intercepts were fit for the two strains, and the two were compared using an F-statistic. A secondary analysis included strain-specific indicators of western diet as an additional predictor. For isolated two-group comparisons, a Mann-Whitney U-test was performed, with P value <0.05 considered statistically significant. Of note, Mann-Whitney U-test on real-time PCR results was performed using ACT values; data are visually represented as mean relative expression ± SE.

RESULTS

Development of emphysema in Apoe−/− mice exposed to an atherogenic high-fat diet. The effect of hypercholesterolemia on the development of emphysema in the Apoe−/− mice subjected to an atherogenic high-fat diet for 10 wk was estimated by measuring the mean linear intercept. At baseline, Apoe+/+ mice fed a Western-type diet for 10 wk demonstrated no difference in mean linear intercept compared with chow-fed Apoe+/+ controls (34.5 ± 2.6 μm vs. 34.5 ± 2.9 μm, respectively). Morphometric analysis demonstrated that Apoe−/− mice fed a Western-type diet for 10 wk developed statistically significant airspace enlargement compared with Apoe−/− mice fed a Western-type diet (41.5 ± 2.8 μm vs. 34.5 ± 2.6 μm, respectively) (Table 1, Fig. 1). Ldlr−/− mice subjected to a Western-type diet for 10 wk did not exhibit emphysematous changes in their lungs (32.7 ± 2.4 μm for chow-fed mice vs. 36 ± 5.4 μm for Western-type diet) (Table 1, Fig. 1). Surface area to unit volume measurements confirmed these findings, with a decrease surface area to unit volume in the Apoe−/− mice fed Western-type diet compared with Apoe+/+ fed West-
LDLr  
Apoe  
Apoe  
Ldlr  
Western-type diet in the mental groups. Macrophage count is also increased with phages were quantified in tissue sections from all experi-
ern-type diet (48.4 ± 3.2 mm$^{-1}$ vs. 58.3 ± 4.4 mm$^{-1}$) (Table 1). Fractional volume measurements did not differ between groups (Table 1).

Inflammatory response to hypercholesterolemia in the lungs of Apoe$^{-/-}$ mice. As provided in Table 2, serum cholesterol is substantially increased with Western-type diet in the Apoe$^{-/-}$ and Ldlr$^{-/-}$ mice, but not in the Apoe$^{+/+}$ mice. To evaluate the impact of hypercholesterolemia on the inflammation in the lungs of Apoe$^{-/-}$ and Ldlr$^{-/-}$ mice, macrophages were quantified in tissue sections from all experimental groups. Macrophage count is also increased with Western-type diet in the Apoe$^{-/-}$ mice, but not in the Ldlr$^{-/-}$ or Apoe$^{+/+}$ mice. The linear regression analysis indicated that Apoe$^{-/-}$ mice macrophage levels increased with serum cholesterol and that the macrophage levels in the Ldlr$^{-/-}$ mice did not increase with serum cholesterol. The estimated intercept and slope parameters (standard errors) for the Apoe$^{-/-}$ mice were 0.15 (1.57) and 0.01 (0.003), whereas for the Ldlr$^{-/-}$ mice were 4.67 (1.07) and −0.001 (0.002). The $F_{2,19}$ statistic for comparing strains was 13.97 ($P < 0.01$). These data indicate that the two strains have substantially different macrophage levels at the same cholesterol levels. When the model was refit with the indicator of diet included as an additional predictor, diet evidenced substantial independent explanatory effects on macrophage count, but serum cholesterol evidenced no independent effect.

Increased expression of MMP-9 and MMP-12 in the lungs of Apoe$^{-/-}$ mice subjected to a Western-type diet. We performed gelatin zymography to determine the activity of MMP-9 in the lungs of Apoe$^{-/-}$ mice subjected to a Western-type diet for 10 wk. Increased activity of MMP-9 was observed in the BAL fluid from Apoe$^{-/-}$ mice (Fig. 2A). MMP-2 activity was detected in the BAL fluid of Apoe$^{-/-}$ and Apoe$^{+/+}$ mice, but levels were not altered. Quantitative RT-PCR analysis demonstrated a trend toward elevation of MMP-9 on mRNA level in the lungs of Apoe$^{-/-}$ mice (Fig. 2A). RT-PCR analysis revealed augmented expression of MMP-12 at the mRNA level in the lungs of Apoe$^{-/-}$ mice ($n = 8$) compared with controls ($n = 8$) (Fig. 2B).

<table>
<thead>
<tr>
<th>Animals</th>
<th>Diet, 10 wk</th>
<th>Mean Linear Intercept, μm</th>
<th>Surface Area/ Unit Volume, mm$^{-1}$</th>
<th>Fractional Volume, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoe$^{+/+}$ mice ($n = 5$)</td>
<td>Chow</td>
<td>34.5 ± 2.9</td>
<td>58.3 ± 5</td>
<td>28.4 ± 2.9</td>
</tr>
<tr>
<td>Apoe$^{-/-}$ mice ($n = 5$)</td>
<td>Western-type</td>
<td>34.5 ± 2.6</td>
<td>58.3 ± 4.4</td>
<td>28.2 ± 2.2</td>
</tr>
<tr>
<td>Apoe$^{-/-}$ mice ($n = 5$)</td>
<td>Chow</td>
<td>38.5 ± 3.2</td>
<td>52.2 ± 4.4</td>
<td>28.6 ± 2.5</td>
</tr>
<tr>
<td>Apoe$^{-/-}$ mice ($n = 6$)</td>
<td>Western-type</td>
<td>41.5 ± 2.8*</td>
<td>48.4 ± 3.2*</td>
<td>29.8 ± 3.7</td>
</tr>
<tr>
<td>LDLr$^{-/-}$ mice ($n = 6$)</td>
<td>Chow</td>
<td>32.7 ± 2.4</td>
<td>61.5 ± 4.8</td>
<td>29.2 ± 5</td>
</tr>
<tr>
<td>LDLr$^{-/-}$ mice ($n = 6$)</td>
<td>Western-type</td>
<td>36 ± 5.4</td>
<td>56.4 ± 7.1</td>
<td>27.5 ± 5.5</td>
</tr>
</tbody>
</table>

Values are means ± SE. Apolipoprotein E-deficient (Apoe$^{-/-}$) mice fed with a Western-type diet exhibit increased mean linear intercept and decreased surface area to volume measurements as compared to Apoe$^{+/+}$ mice fed with the same diet. The low-density lipoprotein receptor-deficient (Ldlr$^{-/-}$) mice do not exhibit such changes, despite similar increases in total serum cholesterol. Fractional volume remains similar among all groups. *$P < 0.05$ versus Apoe$^{+/+}$ Western-type diet.

Fig. 1. Emphysematous changes in the lungs of apolipoprotein E-deficient (Apoe$^{-/-}$) mice subjected to a Western-type diet for 10 wk. Hematoxylin and eosin-stained lung sections. A: Apoe$^{+/+}$ mouse fed a chow diet for 10 wk. B: Apoe$^{+/+}$ mouse fed a Western-type diet (WTD) for 10 wk. C: Apoe$^{-/-}$ mouse fed a chow diet. D: Apoe$^{-/-}$ mouse fed a WTD. E: low-density lipoprotein receptor-deficient (Ldlr$^{-/-}$) mouse fed a chow diet. F: Ldlr$^{-/-}$ mouse fed a WTD.
Activation of TLR signaling pathway in response to hypercholesterolemia in the lungs of Apoe\(^{-/-}\) mice. IRAK is a key regulator in the signaling pathway of TLRs. Once activated, IRAK initiates a cascade of signaling events, ultimately leading to the induction of inflammatory genes such as granulocyte colony-stimulating factor (G-CSF) (13, 52). To evaluate the involvement of the TLR pathway in the development of emphysema in the Apoe\(^{-/-}\) mice, we analyzed the expression of its downstream targets IRAK-1 and G-CSF in the lungs of the Apoe\(^{-/-}\) mice. The analysis of IRAK-1 and G-CSF expression revealed their upregulation in the lungs of Apoe\(^{-/-}\) mice. The analysis of IRAK-1 and G-CSF expression revealed their upregulation in the lungs of Apoe\(^{-/-}\) mice fed a Western-type diet for 10 wk but not in Apoe\(^{+/+}\) mice on the chow diet (Fig. 3, A and B), indicating that the TLR pathway is activated when mice are on the atherogenic diet and not at baseline.

**TLR signaling mediates MMP-9 expression induced in the macrophages of Apoe\(^{-/-}\) mice.** To determine whether Apoe\(^{-/-}\) macrophages were more responsive to TLR activation, peritoneal macrophages from Apoe\(^{-/-}\) mice and their Apoe\(^{+/+}\) controls were treated with various TLR ligands. Activation of TLR4 increased mRNA expression of MMP-9 by eightfold in Apoe\(^{-/-}\) macrophages and by fourfold in Apoe\(^{+/+}\) macrophages (Fig. 3C). Increases in MMP-9 mRNA expression in Apoe\(^{-/-}\) macrophages were also seen after TLR2 activation although not to the extent seen with TLR4 ligand. Interestingly, the TLR3 ligand did not exhibit any effect on MMP-9 expression in either macrophage group, suggesting that the macrophage responsiveness was TLR4/2 specific (Fig. 3C).

**Activation of ERK and JNK, downstream regulators of the TLR signaling pathway, in the lungs of Apoe\(^{-/-}\) mice subjected to a Western-type diet.** Mitogen-activated protein (MAP) kinases such as extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinases (JNK) are activated by the TLR signaling pathway (28). Phosphorylation of ERK and JNK results in the activation of transcription factors regulating inflammatory genes (29). To determine whether Apoe\(^{-/-}\) and

Table 2. **Diet-dependent alterations in cholesterol and pulmonary macrophages are strain dependent**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Total Serum Cholesterol</th>
<th>Macrophage per mm(^2)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Chow diet</td>
<td>Western-type diet</td>
</tr>
<tr>
<td>Apoe(^{+/-}) mice</td>
<td>63 ± 17.78</td>
<td>89 ± 11.9</td>
</tr>
<tr>
<td>Apoe(^{-/-}) mice</td>
<td>335 ± 41.49</td>
<td>610.33 ± 58.19</td>
</tr>
<tr>
<td>LDLr(^{-/-}) mice</td>
<td>225.33 ± 32.01</td>
<td>666.17 ± 60.24</td>
</tr>
</tbody>
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Values are mean ± SE of total serum cholesterol and macrophage count (macrophage per mm\(^2\)) separated by mouse strain and diet type.

Fig. 2. Augmented expression of matrix metalloproteinase-9 (MMP-9) and MMP-12 in the lungs of Apoe\(^{-/-}\) mice subjected to a WTD for 10 wk. A: MMP-9 activity was detected by gelatin zymography in the bronchoalveolar lavage (BAL) fluid of Apoe\(^{-/-}\) mice subjected to a WTD for 10 wk but not in the BAL of Apoe\(^{+/+}\) mice fed with chow diet. There was a trend to increased expression of MMP-9 in lung homogenates in Apoe\(^{-/-}\) mice as detected by real-time PCR. MMP-2 activity was detected in the BAL fluid of Apoe\(^{-/-}\) and Apoe\(^{+/+}\) mice, but activity was not altered between groups. B: MMP-12 expression is elevated in the lungs of Apoe\(^{-/-}\) mice fed a WTD for 10 wk compared with Apoe\(^{+/+}\) controls.
Ldlr⁻/⁻ mice exposed to a Western-type diet have increased levels of phospho-ERK and phospho-JNK in the lung, we analyzed lung lysates by Western blotting. This analysis revealed elevated expression of phospho-ERK and phospho-JNK in the lungs of Apoe⁻/⁻ mice (Fig. 4, A and B). Interestingly, we also observed augmented expression of phospho-ERK in the lungs of Ldlr⁻/⁻ mice, suggesting that activation of ERK potentially occurs early under hypercholesterolemic conditions (Fig. 4 C). In addition, we revealed that the activation of ERK in Apoe⁻/⁻ macrophages by oxidized LDL was further increased by the addition of TLR4 ligand (Fig. 4 D).

DISCUSSION

In the present study, Apoe⁻/⁻ mice subjected to an atherogenic Western-type diet for 10 wk developed emphysematous changes with increased inflammation, enlargement of airspaces, and destruction of alveolar walls. In contrast, Apoe⁺/+ mice and Ldlr⁻/⁻ mice fed an atherogenic diet did not exhibit similar changes. The two strains have substantially different macrophage levels at the same cholesterol levels, with diet demonstrating substantial independent effects on macrophage count. In addition to observing TLR2/4 pathway activation in the lungs of Apoe⁻/⁻ mice fed with Western-type diet, we also detected increases in two major elastinolytic proteases, including augmented activity of MMP-9 and expression of MMP-12. Macrophages from Apoe⁻/⁻ mice were sensitive to TLR4 activation, the effects of which were augmented in the presence of oxidized LDL. The subsequent inflammatory cell recruitment, mediated by G-CSF production, and protease production likely contribute to the observed destruction of the lung extracellular matrix in the Apoe⁻/⁻ mice.

Studies have suggested that COPD is a systemic disease rather than an independent disease state (18). Atherosclerosis is one of the leading causes of mortality in COPD (2). The major hallmark of both diseases is the presence of chronic inflammation with the recruitment of macrophages and lymphocytes (5). It has been demonstrated that murine models of smoke-induced emphysema exhibit a marked increase in the number of macrophages and neutrophils and have augmented myeloperoxidase activity (20). Arunachalam and colleagues (3) recently demonstrated that ApoE-deficient mice exposed to cigarette smoke manifest an increased inflammation response, characterized by infiltration of macrophages and neutrophils into the lung and increased oxidative stress. Our data demonstrate that, even in the absence of smoking, an atherogenic diet fed to Apoe⁻/⁻ mice induces increased inflammation through...
TLR4 activation, with subsequent augmented protease production that results in emphysema. These results are unique to the Apoe\(^{-/-}\) mice, as the Ldlr\(^{-/-}\) mice, which achieve the same level of hyperlipidemia, do not develop pulmonary inflammation and lung destruction. Hypercholesterolemia alone, as is seen in the Ldlr\(^{-/-}\) mice, is not sufficient to induce increased inflammation and protease production, despite the activation of MAP kinases in the lung. It is possible that diet serves as a better proxy for serum cholesterol than a single, possibly labile, measure of serum cholesterol. However, this may also indicate that the effect of diet in the Apoe\(^{-/-}\) mice is not solely through serum cholesterol levels.

The increased susceptibility to emphysema formation in Apoe\(^{-/-}\) mice compared with Ldlr\(^{-/-}\) mice is likely attributed to additional alterations in cholesterol efflux present within the Apoe\(^{-/-}\) macrophages (53, 65). ApoE promotes macrophage cholesterol efflux (53, 65) through the ABCA-1 and ABCG-1 cell surface transporters, ultimately initiating the formation of HDL particles (53). Interestingly, Apcal\(^{-/-}\) and Abcg1\(^{-/-}\) mice manifesting abnormal cholesterol efflux exhibit pulmonary inflammation (6, 9). Macrophages from these mice exhibit increased expression of inflammatory genes via TLR4 signaling, suggesting a link between alterations in cholesterol efflux and lung inflammation through TLR4 signaling (63).

In the present study, we provide evidence for the role of TLR4 signaling in the development of emphysema in an atherosclerosis animal model devoid of cigarette smoke exposure. The TLR4 signaling pathway plays a crucial role in the regulation of the immune response in atherosclerosis and in various lung-associated pathologies (7, 12) and is regulated by oxidized LDL (15, 39, 61). TLR4 activation reduces the expression of the genes involved in cholesterol transport and metabolism, ultimately causing a pathological lipid accumulation in macrophages (14). Additionally, free cholesterol accumulation in macrophage membranes activates TLRs, resulting in downstream MMP induction in atherosclerosis (51).

Numerous studies have shown that activation of TLR2 or TLR4 leads to the recruitment of IRAK-1, IRAK-4, and TNF receptor-associated factor 6 (24). This association results in the activation of ERK and JNK, MAP kinases involved in both TLR signaling and MMP production (21, 38). The present study provides evidence for activation of the TLR4 signaling pathway in the lungs of Apoe\(^{-/-}\) mice fed an atherogenic diet. Activation of this pathway is indicated in the analysis of downstream targets of TLR4 in the lung, including IRAK-1 and G-CSF. In vitro experiments on macrophages from Apoe\(^{+/+}\) and Apoe\(^{-/-}\) treated with TLR4 ligand (100 ng/ml) and oxidized LDL (100 μg/ml). Activation of ERK in Apoe\(^{-/-}\) macrophages treated with oxidized LDL was augmented by the addition of TLR4 ligand.

Fig. 4. Western blot analysis of ERK and JNK phosphorylation in the lungs of mice. A–B: Western blot analysis of phospho-ERK (ph-ERK) and phospho-JNK (ph-JNK) in the lungs of Apoe\(^{-/-}\) mice after 10 wk of WTD. Densitometric analysis of the signal was performed to determine ph-ERK/total ERK and JNK/actin ratios. C: Western blot analysis of ph-ERK in the lungs of Ldlr\(^{-/-}\) mice fed a WTD for 10 wk. D: peritoneal macrophages from Apoe\(^{-/-}\) and Apoe\(^{+/+}\) treated with TLR4 ligand (100 ng/ml) and oxidized LDL (100 μg/ml). Activation of ERK in Apoe\(^{-/-}\) macrophages treated with oxidized LDL was augmented by the addition of TLR4 ligand.

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BAL fluid from Apoe−/− mice fed with Western-type diet showed clear increase in MMP-9 activity, this result was not seen at the mRNA expression level in whole lung homogenates. The whole lung homogenates contain cell types that do not express MMP-9 and likely alter the amount of difference seen between the groups; the zymography results provide a more accurate assessment of the augmented MMP-9 activity at the alveolar level. Our laboratory has demonstrated that the transgenic overexpression of MMP-9 in macrophages causes spontaneous emphysema attributable to elastin degradation (19), which appears in contrast to studies demonstrating that an MMP-9-deficient mouse still develops smoke-induced emphysema (4). However, in the MMP-9-deficient mice, the presence of other proteases, including MMP-12, is potentially sufficient to destroy the lung. Therefore, it is likely that the combined upregulation of MMP-9 and MMP-12 in the lungs of Apoe−/− mice causes disruption of the lung extracellular matrix and ultimately contributes to the observed emphysematous changes seen in this model.

These findings have significant public health implications in the age of increasing incidence of obesity, atherosclerosis, and emphysema. It is already well established that patients with COPD have increased cardiovascular morbidity and mortality, especially in a younger cohort (47). However, the reverse is also true, with the development of COPD in patients with atherosclerosis substantially reducing cardiovascular disease survival rates (35). In patients with atherosclerosis, even a decline in forced expiratory volume in 1 s, independent of smoking history and other atherosclerosis risk factors, is associated with early mortality from cardiovascular causes (11, 48). Leptin, recently atherosclerosis risk factors, is associated with increased inflammation in lungs of atherosclerosis-prone mice by cigarette smoke: implications in comorbidities of COPD. J Inflamm 7: 34, 2010.


