Histamine Deficiency Decreases Atherosclerosis and Inflammatory Response in Apolipoprotein E Knockout Mice Independently of Serum Cholesterol Level

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Objective—Histamine and histamine receptors are found in atherosclerotic lesions, and their signaling and subsequent proatherogenic or proinflammatory gene expression are involved in atherogenesis. In the present study, we generated apolipoprotein E (apoE) and histamine synthesizing histidine decarboxylase double knockout (DKO) mice on a C57BL/6j (wild-type mice) background to clarify the roles of histamine in atherosclerosis.

Methods and Results—Wild-type, apoE knockout (KO), and DKO mice were fed a high-cholesterol diet to analyze hyperlipidemia-induced atherosclerosis. Compared with wild-type mice, apoE-KO mice showed increased expression of histamine and its receptors, corresponding to increased atherosclerotic lesion areas and expression of inflammatory regulators, such as nuclear factor-κB, scavenger receptors, inflammatory cytokines, and matrix metalloproteinases. Histamine deficiency after deletion of histidine decarboxylase reduced atherosclerotic areas and expression of a range of the inflammation regulatory genes, but serum cholesterol levels of DKO mice were higher than those of apoE-KO mice.

Conclusion—These results indicate that histamine is involved in the development of atherosclerosis in apoE-KO mice by regulating gene expression of inflammatory modulators, an action that appears to be independent of serum cholesterol levels. In addition to acute inflammatory response, histamine participates in chronic inflammation, such as hyperlipidemia-induced atherosclerosis, and might be a novel therapeutic target for the treatment of atherosclerosis. (Arterioscler Thromb Vasc Biol. 2011;31:800-807.)

Key Words: histamine ■ histidine decarboxylase ■ hyperlipidemia-induced atherosclerosis ■ inflammation ■ matrix metalloproteinase

Recent evidence has emerged concerning inflammatory mechanisms of the initiation and progression of atherosclerosis.1-2 Histamine, one of the classical inflammatory mediators, is synthesized from l-histidine by a rate-limiting enzyme, histidine decarboxylase (HDC). Histamine is released from mast cells and mediates type I hypersensitivity via histamine receptor H1 (HH1R),3 and histamine produced by enterochromaffin-like cells induces gastric acid secretion from parietal cells via histamine receptor H2 (HH2R).4 In the field of cardiovascular pathology, accumulation of activated mast cells and histamine in the coronary adventitia has been implicated in progression of plaque rupture and acute coronary syndrome.5-7 In addition, several epidemiological studies have reported an enhancement of atherosclerosis in the patients of allergy or increased blood histamine.8-10 Together, these suggest a possible involvement of histamine in the pathogenesis of atherosclerosis and related disorders.

Previously, we demonstrated that HDC-knockout (KO) mice showed reduced neointimal formation induced by ligation of the carotid artery or cuff replacement of the femoral artery.11 Because histamine stimulates smooth muscle cells (SMCs) to proliferate and SMCs predominantly express HH1R,12 the neointimal formation, which consists of SMCs, is suggested to be an HH1R-mediated response.11 Although ligation- or cuff-induced intimal hyperplasia mimics diffuse intimal thickening as a precursor lesion of atherosclerosis,13 it is quite different from established atherosclerosis, in which accumulation of lipid-laden macrophages has a unique his-

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ology. Interestingly, infiltrating macrophages in the atherosclerotic lesions express HDC as a source of histamine in human carotid arteries and the aortas of apolipoprotein E (apoE)–KO mice.\textsuperscript{11,14,15} HDC expression in human monocytes is upregulated during macrophage differentiation, which corresponds to a switch of the histamine receptor profile from HH2R predominance in monocytes to HH1R dominance in macrophages.\textsuperscript{16,17} Together, these results indicate that both histamine production and response is present in the cells, constituting atherosclerotic lesions and that macrophage-derived histamine could regulate atherogenic response.\textsuperscript{18} However, the roles of histamine in the process of hyperlipidemia-induced atherosclerosis still remain unclear.

To further the examination, we generated HDC and apoE double-knockout (DKO) mice to investigate the roles of histamine in hyperlipidemia-induced atherosclerosis. The expression of inflammatory cytokines, scavenger receptors (SRs), matrix metalloproteinases (MMPs), and nuclear factor-\(\kappa\)B (NF-\(\kappa\)B), which regulate inflammatory response in the atherosclerotic lesions, was studied by reverse transcription–polymerase chain reaction (RT-PCR), Western blotting, and immunohistochemistry. In addition, we studied the effects of histamine on serum cholesterol.

**Materials and Methods**

**Animals**

We generated DKO mice by crossing apoE-KO mice (Jackson Laboratory, Bar Harbor, ME) with previously generated HDC-KO mice.\textsuperscript{10} Male mice were weaned at 8 weeks of age onto a high-cholesterol diet (HcD) consisting of 1.25% cholesterol, 15% lard, and 0.5% sodium cholate (Oriental Yeast Co, Tokyo, Japan) and were maintained on this diet for 12 weeks. Another group was maintained to 23 weeks and 33 weeks of age on a normal chow diet (NcD). Wild-type (WT) C57BL/6j mice (Charles River, Yokohama, Japan) were used for control groups. Each experimental group included at least 10 mice. Animals were maintained on a 12-hour light/dark cycle. All protocols were approved by the Ethics Committee of Animal Care and Experimentation, University of Occupational and Environmental Health, and were performed according to the institutional guidelines for animal experiments and according to Law 105 and Notification 6 of the Japanese government.

**Assessment of Atherosclerosis and Immunohistochemistry**

The aortas were cut open longitudinally and fixed with 10% neutral buffered formalin for 24 hours. Then the aortas were stained with Oil Red \(O\) stain. \textit{En face} images of the aortas were captured with a digital camera. Oil Red \(O\)–stained area relative to whole surface area was calculated using NIH Image software. For histological analysis, every 10-step sections of 1-cm length ascending aortas from the aortic valve (4-\(\mu\)m-thick step sections: 1500 sections/aorta) were stained with hematoxylin and eosin stain. After scanning using a digital camera, the intima/media ratio and intimal plaque area were evaluated using NIH Image.\textsuperscript{11,20} Immunostaining was carried out (Envision kit, Dako, Tokyo, Japan) on the paraffin sections using antibodies EPOS anti-\(\alpha\)-smooth muscle actin (clone 1A4, \(\times\)100, Dako, Carpenteria, CA), macrophages (Mac-3 clone M3/84, \(\times\)50, BD Bioscience Pharmingen, Tokyo, Japan), HDC (rabbit polyclonal, \(\times\)100; Progen Biotechnik, Heidelberg, Germany), and histamine (rabbit polyclonal, \(\times\)100; Progen Biotechnik) as previously described.\textsuperscript{15} Immunolocalization of NF-\(\kappa\)B was also studied in the atherosclerotic lesions (rabbit polyclonal, \(\times\)2000, Abcam).

**Lipoprotein Analysis**

After mice were starved for 7 hours, blood was collected, and the serum was analyzed by high-performance liquid chromatography (Skylight Biotech, Akita, Japan).\textsuperscript{21}

**Real-Time Polymerase Chain Reaction**

Expression of mRNA in the liver and aortas was quantified by real-time RT-PCR using TaqMan quantitative PCR analysis. The genes investigated and primers for PCR are listed in Table 1.

**Measurement of Histamine and Monocyte Chemoattractant Protein-1 in Serum and Aortic Tissue**

The aortic and serum levels of monocyte chemoattractant protein-1 (MCP-1) and histamine were measured by ELISA (R&D Systems and Immunotech, Marseille, France). The aortas were homogenized in 0.2 N \(\text{HClO}_4\) buffer (100 \(\mu\)L/10 \(\mu\)g tissue), and the supernatants were collected by centrifugation (10,000 \(g\) for 5 minutes at 4°C). After neutralization by addition of an equal volume of 1 mol/L potassium borate (pH 9.25) and measurement of protein concentration, the supernatants were subjected to ELISA.

**Western Blotting**

The aortic expression of class A SR (SR-A), CD36 (rabbit polyclonal, \(\times\)1000; Santa Cruz Biotechnology), and NF-\(\kappa\)B (rabbit polyclonal, \(\times\)2000, Abcam) proteins was studied by Western blotting.

**Statistical Analysis**

ANOVA was applied to determine statistical differences, and a probability value of less than 0.05 was taken to be significant.

**Results**

**General Phenotypes of DKO Mice**

The body weight of apoE-KO mice was not increased, and that of WT and DKO mice was increased after NcD for 33 weeks (Supplemental Figure IA, available online at http://atvb.ahajournals.org). Both systolic and diastolic blood pressure was increased in apoE-KO mice compared with WT mice. In DKO mice, blood pressure was decreased to the level of WT mice (Supplemental Figure IB). White blood cell counts were not different among WT, apoE-KO, and DKO mice, but percentages of neutrophiles and lymphocytes were increased in apoE-KO mice. Very few basophiles were observed in the peripheral blood from WT, apoE-KO, and DKO mice (Supplemental Table I). No infectious diseases or other pathological conditions were observed during the experiments in the mice.

**Serum Cholesterol Levels in DKO Mice**

On feeding with NcD for 23 to 33 weeks or with HcD for 12 weeks from the age of 8 weeks, apoE-KO and DKO mice became hyperlipidemic, with increased total cholesterol (T-cho), very-low-density lipoprotein (VLDL) cholesterol, and...
Table 1. Primers Used for Real-Time PCR

<table>
<thead>
<tr>
<th>Gene*</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>TaqMan Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD106 (VCAM-1)</td>
<td>CTCATTCCCTGAGATGATTAAC</td>
<td>CCTAGGCAAGATGATTCTG</td>
<td>TGACACCTGCTGCTGCTG</td>
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<tr>
<td>CD36</td>
<td>AGGTCTTACACATCAAGGCTGTTT</td>
<td>AAACGAGTGATGACCTAAGGA</td>
<td>ACTACGACTGAGACAGAC</td>
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<tr>
<td>CD54 (ICAM-1)</td>
<td>CACAGCATGATGATGATGATGATG</td>
<td>ACCAGGCTGATGATGATGATG</td>
<td>TGATACGACTGAGACAGAC</td>
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<tr>
<td>HDC</td>
<td>CAAAGTGCAGGAGCTGATAC</td>
<td>GTTGAGGAGCTGATGATG</td>
<td>CTACGACTGAGACAGAC</td>
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<td>HH1R</td>
<td>CTTGATGAGGAGCTGATGATG</td>
<td>TTTGAGGAGCTGATGATG</td>
<td>CTACGACTGAGACAGAC</td>
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<tr>
<td>HH2R</td>
<td>GCTTCTCCTCGGACAGATGATG</td>
<td>TTTGAGGAGCTGATGATG</td>
<td>CTACGACTGAGACAGAC</td>
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<td>IL1-β</td>
<td>TGGAGGAGCTGATGATG</td>
<td>TTTGAGGAGCTGATGATG</td>
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<td>IL6</td>
<td>AGGTATAAGGAGCTGATGATG</td>
<td>TTTGAGGAGCTGATGATG</td>
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<td>MCP-1</td>
<td>GCTCTCCTCGGACAGATGATG</td>
<td>TTTGAGGAGCTGATGATG</td>
<td>CTACGACTGAGACAGAC</td>
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<td>MMP-2</td>
<td>CTTTCCTGCTCGGACAGATGATG</td>
<td>TTTGAGGAGCTGATGATG</td>
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<td>MMP-3</td>
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<td>MMP-9</td>
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<td>PDGF-β</td>
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<td>SR-BI</td>
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<tr>
<td>TNFR2</td>
<td>TGGAGGAGCTGATGATG</td>
<td>TTTGAGGAGCTGATGATG</td>
<td>CTACGACTGAGACAGAC</td>
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</table>

18s rRNA TaqMan Ribosomal RNA Control Reagents VIC Probe (Applied Biosystems, catalog no. 4308329)

* iNOS indicates inducible nitric oxide synthase; PDGF, platelet-derived growth factor; TNFR, tumor necrosis factor receptor.

Table 2. HPLC Analysis of Serum Lipoproteins

<table>
<thead>
<tr>
<th>Lipoprotein</th>
<th>Diet</th>
<th>Week</th>
<th>WT</th>
<th>ApoE-KO</th>
<th>DKO</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-cho (mg/dL)</td>
<td>NcD</td>
<td>23</td>
<td>68.3±2.7</td>
<td>325.0±35.4</td>
<td>428.2±24.8</td>
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<tr>
<td>NcD</td>
<td>33</td>
<td>78.3±3.7</td>
<td>365.5±52.0</td>
<td>496.1±37.0</td>
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<tr>
<td>HcD</td>
<td>12</td>
<td>166.5±8.4</td>
<td>470.1±61.1</td>
<td>633.5±50.8</td>
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<tr>
<td>VLDL (mg/dL)</td>
<td>NcD</td>
<td>23</td>
<td>3.9±0.6</td>
<td>137.7±22.8</td>
<td>225.7±14.1</td>
</tr>
<tr>
<td>NcD</td>
<td>33</td>
<td>6.1±0.8</td>
<td>208.5±34.4</td>
<td>283.4±24.4</td>
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<tr>
<td>HcD</td>
<td>12</td>
<td>63.7±6.1</td>
<td>284.8±39.1</td>
<td>381.4±30.9</td>
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<tr>
<td>LDL (mg/dL)</td>
<td>NcD</td>
<td>23</td>
<td>7.5±0.3</td>
<td>94.5±10.3</td>
<td>153.6±10.5</td>
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<tr>
<td>NcD</td>
<td>33</td>
<td>9.0±0.6</td>
<td>107.5±17.6</td>
<td>161.7±10.5</td>
<td></td>
</tr>
<tr>
<td>HcD</td>
<td>12</td>
<td>39.2±2.6</td>
<td>108.4±19.9</td>
<td>165.3±10.7</td>
<td></td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td>NcD</td>
<td>23</td>
<td>56.9±2.0</td>
<td>26.2±2.8</td>
<td>36.6±3.6</td>
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<tr>
<td>NcD</td>
<td>33</td>
<td>63.7±2.8</td>
<td>28.3±1.4</td>
<td>35.6±2.2</td>
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<tr>
<td>HcD</td>
<td>12</td>
<td>60.9±3.1</td>
<td>23.0±2.1</td>
<td>32.5±4.2</td>
<td></td>
</tr>
</tbody>
</table>

T-cho indicates total cholesterol; VLDL, very-low-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein.

* P<0.05 vs WT.
† P<0.05 vs apoE-KO.

Induction of Histamine and Histamine Receptors by Hyperlipidemia

Serum histamine levels were increased in apoE-KO mice compared with WT mice during the ages of 23 to 33 weeks (Table 2). Furthermore, compared with apoE-KO mice, DKO mice showed higher cholesterol levels in all fractions, but HDL cholesterol was moderately increased in DKO mice.

Suppression of Hyperlipidemia-Induced Atherosclerosis in DKO Mice

En face analysis demonstrated that atherosclerotic lesion area was markedly increased during the age of 23 to 33 weeks in DKO mice fed the high-fat diet compared with WT and apoE-KO mice. Real-time RT-PCR showed increased HDC expression in atherosclerotic aortas of apoE-KO mice fed the high-fat diet. Expression of HH1R, HH2R, and HH3R in atherosclerotic aortas was increased in apoE-KO mice compared with WT mice after HcD feeding, whereas it was significantly decreased in DKO mice (Figure 1D and Supplemental Figure II).
apoE-KO mice fed NcD, whereas it was much less in DKO mice (40% of apoE-KO mice) (Figure 2A). After mice were fed HcD for 9 or 12 weeks, atherosclerotic lesion area in DKO mice was also 40% of that of apoE-KO mice (Figure 2B). The atherosclerotic lesions of apoE-KO mice included Mac-3-positive macrophages (foam cells) and a lesser number of α-SMA–positive SMCs. The macrophages were positive for HDC and histamine in apoE-KO mice (Figure 2C). In addition to the Mac-3-positive macrophages, a few CD3-positive T lymphocytes infiltrated in the atherosclerotic intima (data not shown). The T cell counts were increased in apoE-KO and DKO mice compared with WT mice, but they were not different between apoE-KO and DKO mice (Supplemental Table II).

Because mast cells in HDC-KO mice are decreased in number and show abnormal morphology and reduced granular content, the mast cells in the atherosclerotic aortas were studied by toluidine blue stain. Mast cells were not observed in the atherosclerotic intima, but a few cells were detected in the adventitia, and no significant differences in these cell numbers were noted among WT, apoE-KO, and DKO mice (data not shown).

In 33-week-old mice fed NcD, intima/media ratio and intimal lesion area were significantly reduced by 60% in DKO mice (Figure 3).
expression of inflammatory cytokines or growth factors and their receptors, such as tumor necrosis factor receptor, interleukin-1 receptor (IL1R), IL-1β, IL-6, platelet-derived growth factor β chain, and MCP-1, was increased in apoE-KO mice compared with WT mice and decreased in DKO mice compared with apoE-KO mice. SRs, including SR-A, SR-BI, CD36, and lectin-like oxidized LDLR-1 (LOX-1), were increased in apoE-KO mice and decreased in DKO mice. The expression of these SRs genes was significantly downregulated in DKO mice compared with apoE-KO mice (Figure 3A and Supplemental Figure III). The levels of protein expression of SR-A and CD36 were correlated to those of mRNA expression (Figure 3B). In the liver, mRNA expression of SR-BI was moderately decreased in DKO mice, but that of LDLR was increased compared with apoE-KO mice (Figure 3B). Serum and aortic tissue MCP-1 protein levels were increased in apoE-KO mice compared with WT mice but decreased in DKO mice. MMP expression was also increased in atherosclerotic plaques of apoE-KO mice, except for MMP-9, and was decreased in DKO mice. The expression of NF-κB was studied by Western blotting (B) and immunohistochemistry (C). In apoE-KO mice, NF-κB expression was increased and localized in the nuclei of the macrophages. Both expression and nuclear localization were decreased in DKO mice (Figure 5A). Inducible nitric oxide synthase, expression of which is regulated by HH1R signaling in vascular SMCs, was enhanced in apoE-KO mice and reduced in DKO mice (Supplemental Figure III).

Suppression of Inflammatory Response in Atherosclerotic Aortas of DKO Mice

Real-time RT-PCR revealed that expression of LDL receptor (LDLR) was decreased in both apoE-KO and DKO compared with WT mice but was further decreased in DKO mice compared with apoE-KO mice. SRs, including SR-A, SR-BI, CD36, and lectin-like oxidized LDLR-1 (LOX-1), were increased in apoE-KO mice and decreased in DKO mice after feeding with HcD for 12 weeks. Values were normalized by 18S rRNA expression and are presented as mean±SE. A, ICAM-1 and VCAM-1 expression was increased in atherosclerotic aortas of apoE-KO mice but significantly decreased in DKO mice. B, Aortic MCP-1 content in DKO mice was lower than that in apoE-KO mice in 12 weeks of HcD feeding. C, Serum MCP-1 was increased in apoE-KO and DKO mice fed HcD from 23 to 33 weeks of age. After HcD feeding for 9 and 12 weeks, serum MCP-1 of both apoE-KO and DKO mice were increased compared with that of WT mice. Tissue MCP-1 was normalized by tissue weight. The mRNA expression was normalized by 18S rRNA expression and is presented as mean±SE. *P<0.05, **P<0.01, ***P<0.001 vs WT; #P<0.05, ##P<0.01 vs apoE-KO mice.

Decreased Expression and Nuclear Localization of NF-κB in Atherosclerotic Aortas in DKO Mice

One of the key regulators of inflammation, transcriptional factor NF-κB expression in the aortas, was evaluated by Western blotting and immunohistochemistry. In the atherosclerotic aortas of mice fed HcD for 12 weeks, expression was markedly increased in apoE-KO mice and decreased in DKO mice (Figure 5B). As shown by immunostaining, nuclear localization of NF-κB in the infiltrated macrophages, detected in apoE-KO mice, was decreased in DKO mice (Figure 5C).
In the present study, we demonstrated that serum and aortic histamine contents and histamine receptor expression increased as hyperlipidemia-induced atherosclerosis developed in apoE-KO mice. In histamine-deficient DKO mice, atherosclerotic lesion was significantly attenuated despite the higher cholesterol levels. The expression of a range of proatherogenic cytokines, SRs, adhesion molecules, and MMPs in the aortas was reduced in DKO mice. Expression and activation of NF-κB, one of the key inflammatory regulators, was also decreased in DKO mice. Therefore, these results indicate that histamine promotes atherosclerosis independently of serum cholesterol levels but depending on gene regulation of inflammatory response.

Regulation of Atherosclerosis and Serum Lipid by Histamine

In both liver and atherosclerotic aortas, SR-BI expression were reduced in DKO compared with apoE-KO mice. HDL cholesterol receptor SR-BI regulates reverse cholesterol transport from peripheral atherosclerotic lesions to the liver, and deletion of SR-BI in apoE-KO mice accelerates proatherogenic hypercholesterolemia and atherosclerosis.17 Therefore, decreased expression of SR-BI is responsible in part for the increased cholesterol levels in DKO mice. In contrast, LDLR expression in the liver was moderately increased and that in the aortas was decreased in DKO mice. Because LDLR is expressed predominantly in the liver and LDLR-deficient mice show proatherogenic lipid profile because of reduced hepatic clearance of LDL and very-low-density lipoprotein,30,31 it is probable that LDLR-mediated clearance of serum cholesterol would not, at least, be impaired in DKO mice.

As expected, HDL cholesterol levels in DKO mice were higher than those in apoE-KO mice. The higher serum HDL cholesterol levels might partly participate in the reduction of atherosclerosis in DKO mice. Although the net effect of histamine deficiency on apoE-KO mice is attenuation of atherosclerosis with increased very-low-density lipoprotein, LDL, and HDL cholesterol, the exact mechanism(s) by which histamine regulates cholesterol metabolism is still unknown. Recently, however, we suggested that hepatic cholesterol accumulation is regulated by histamine signaling in the liver,32 and therefore histamine actions mediated through histamine receptors expressed in tissues, including the artery and liver, probably regulate cholesterol metabolism.

On the other hand, SR-A, CD36, LOX-1, and SR-BI, which are the receptors for oxidized LDL and mainly expressed in atherosclerotic lesions to promote atherosclerosis,22–25 were decreased in DKO mice. Partially supporting the present results, LOX-1 expression in monocytes is upregulated by histamine-mediated signal through HH2R.17 Therefore, suppressed influx of modified LDL in aortas by histamine deficiency is implicated in reduced atherosclerosis progression in DKO mice in spite of increased serum cholesterol levels. In fact, atherosclerosis, which is enhanced in LDLR-KO or apoE-KO mice, is reduced by deficiency of LOX-1, SR-A, and CD36 expression.33,34 Histamine is able to enhance cholesterol influx in peripheral tissues, resulting in the accumulation of cholesterol in lipid-laden cells in the aortas to accelerate atherosclerosis.

Regulation of Inflammatory Response by Histamine

Our study showed that proatherogenic cytokines and other molecules (such as IL-1β, IL-6, platelet-derived growth factor-BB, inducible nitric oxide synthase, and MCP-1) which regulate inflammatory response in the atherosclerotic lesions, were markedly decreased along with attenuation of hyperlipidemia-induced atherosclerosis in DKO mice. Because influx of modified LDL into the atherosclerotic lesions also accelerates the inflammatory responses to the progress of atherosclerosis,35,36 the decreased cholesterol influx contributes to the decreased inflammation to reduce atherosclerotic lesions in DKO mice.

Among those inflammatory factors, our previous studies showed histamine regulation of MCP-1 in relation to atherogenesis.15,37,38 Histamine stimulates monocytes to express MCP-1 and its receptor chemokine (c-c motif) receptor 2 (CCR2), and it stimulates endothelial cells to upregulate ICAM-1 and VCAM-1 expression,37 which are upregulated at atherosclerosis-prone sites in apoE-KO mice.39 The expression of MCP-1 is also upregulated by granulocyte-macrophage colony-stimulating factor, which enhances the production of histamine via HDC expression of monocytels.15,38 These data suggest that histamine modulates monocyte migration from peripheral blood via upregulation of MCP-1/CCR2 and adhesion molecules and that histamine is involved in an inflammatory network in the atherosclerotic lesion.18 Actually, in the present study, the serum and aortic MCP-1 expression in DKO mice was significantly lower than that in apoE-KO mice. In addition, the aortic expression of endothelial and monocytic adhesion molecules, including ICAM-1 and VCAM-1, was induced in apoE-KO mice but significantly reduced in DKO mice. Because the increased expression of MCP-1 and adhesion molecules plays a central role in the progression and destabilization of established atherosclerosis in apoE-KO mice,40 these data indicate that the antiatherogenic nature of DKO mice could, at least partially, be attributed to the downregulation of histamine-induced expression of MCP-1 and adhesion molecules.

Regulation of MMP Expression and Intimal Remodeling by Histamine

Transgenic expression or KO of MMP genes has been very often introduced in apoE-KO mice or another animal to investigate the relation between atherosclerosis and arterial matrix degradation.41–43 Of special interest, the effects of MMP-9 and MMP-12 are well studied because of its elastolytic activity to destroy the arterial media for the progression of atherosclerosis. Indeed, we previously reported that MMP-12 plays an essential role in the invasion of macrophages into hypercholesterolemia-induced atherosclerotic foci in MMP-12 transgenic rabbits by disruption of elastic fibers.20 The current data concerning MMPs indicate that
histamine participates in tissue remodeling in hyperlipidemia-induced atherosclerosis via the expression of MMP-2, -3, -9, and -12. Although histamine is not able to directly induce these expression of these MMPs in cultured macrophages and SMCs (data not shown), the expression of MMPs is regulated by a complicated inflammation network including histamine in the atherosclerotic lesions. 19 It is of note that oxidized LDL–induced expression of MMP-2 and -9 in atherosclerotic lesions of LDLR-KO mice is mediated though activation of LOX-1, 44 because our previous study showed that histamine upregulates LOX-1 expression in monocytes. 17 The histamine-LOX-1-MMP axis may be present in atherosclerotic lesions to modulate extracellular matrix metabolism.

Regulation of NF-κB Signaling in Inflammatory Responses by Histamine
NF-κB signaling is critical for atherogenesis because it regulates vascular inflammatory responses, and activated NF-κB has demonstrated in atherosclerotic lesions. 45,46 Importantly, all the genes whose expression was decreased in DKO mice are targets of the transcriptional factor NF-κB in the atherosclerotic lesion. 45 Because the expression and nuclear localization of NF-κB were decreased in DKO mice, these data indicate that histamine regulates NF-κB signaling in atherogenesis. In conclusion, the present study indicates that the inflammatory response induced by histamine is an important regulator of atherosclerosis induced by high serum cholesterol. Therefore, histamine could be a relevant therapeutic target in the treatment of atherosclerosis.

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Disclosures
No competing financial interests exist.

References
22. Gough PJ, Greaves DR, Suzuki H, Hakkinen T, Hiltunen MO, Turunen H. Histamine-induced metalloproteinase activity is upregulated by a complicated inflammation network including histamine in the atherosclerotic lesions. 19 It is of note that oxidized LDL–induced expression of MMP-2 and -9 in atherosclerotic lesions of LDLR-KO mice is mediated though activation of LOX-1, 44 because our previous study showed that histamine upregulates LOX-1 expression in monocytes. 17 The histamine-LOX-1-MMP axis may be present in atherosclerotic lesions to modulate extracellular matrix metabolism.

Regulation of NF-κB Signaling in Inflammatory Responses by Histamine
NF-κB signaling is critical for atherogenesis because it regulates vascular inflammatory responses, and activated NF-κB has demonstrated in atherosclerotic lesions. 45,46 Importantly, all the genes whose expression was decreased in DKO mice are targets of the transcriptional factor NF-κB in the atherosclerotic lesion. 45 Because the expression and nuclear localization of NF-κB were decreased in DKO mice, these data indicate that histamine regulates NF-κB signaling in atherogenesis. In conclusion, the present study indicates that the inflammatory response induced by histamine is an important regulator of atherosclerosis induced by high serum cholesterol. Therefore, histamine could be a relevant therapeutic target in the treatment of atherosclerosis.

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The authors thank Dr Masao Kimoto in the Faculty of Medicine, Saga University, for his critical suggestions and also to thank Hiroko Isakai, Hana Nishimura, Naoko Une, and Tomoko Shimia for their technical assistance.

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Disclosures
No competing financial interests exist.
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Histamine Deficiency Decreases Atherosclerosis and Inflammatory Response in Apolipoprotein E Knockout Mice Independently of Serum Cholesterol Level

Ke-Yong Wang, Akihide Tanimoto, Xin Guo, Sohsuke Yamada, Shohei Shimajiri, Yoshitaka Murata, Yan Ding, Masato Tsutsui, Seiya Kato, Teruo Watanabe, Hiroshi Ohtsu, Ken-Ichi Hirano, Kimitoshi Kohno and Yasuyuki Sasaguri

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Supplemental Fig. I.

A  Body weight

- - - - WT
- - - - apoE-KO
- - - - DKO

# p<0.05 vs. WT

B  Blood pressure

0 20 40 60 80 100 120

SBP

DBP

WT  apoE-KO  DKO

* p<0.05 vs. WT
# p<0.05, ## p<0.001
Supplemental Fig. II.

* p<0.05 vs. WT
# p<0.05
Supplemental Fig. III.

A

LOX-1/18S

WT apoe DKO

B

IL1R1/18S

WT apoe DKO

C

TNFR2/18S

WT apoe DKO

D

MMP2/18S

WT apoe DKO

E

MMP3/18S

WT apoe DKO

F

iNOS/18S

WT apoe DKO
### Table I.

#### Peripheral Blood Cell Count

<table>
<thead>
<tr>
<th></th>
<th>WBC (counts/µL)</th>
<th>NEU (%)</th>
<th>EOS (%)</th>
<th>BASO (%)</th>
<th>MONO (%)</th>
<th>LY (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WT</strong></td>
<td>10992±1599</td>
<td>12.00±2.10</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>1.75±0.75</td>
<td>86.25±2.13</td>
</tr>
<tr>
<td><strong>ApoE-KO</strong></td>
<td>9125±1099</td>
<td>35.83±5.46*</td>
<td>0.16±0.16</td>
<td>0.00±0.00</td>
<td>2.66±0.98</td>
<td>61.33±5.80*</td>
</tr>
<tr>
<td><strong>DKO</strong></td>
<td>10296±1839</td>
<td>25.60±7.58</td>
<td>0.40±0.40</td>
<td>0.00±0.00</td>
<td>3.00±1.05</td>
<td>71.00±8.29</td>
</tr>
</tbody>
</table>

NEU, Neutrophile; EOS, eosinophile; BASO, Basophile; MONO, monocyte; LY, Lymphocyte
* p<0.05 vs. WT n=5

#### Femoral Bone Marrow Cell Counts

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>apoE-KO</th>
<th>DKO</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total cell counts (x10^7/femur)</strong></td>
<td>1.1±0.1</td>
<td>1.4±0.1</td>
<td>1.3±0.1</td>
</tr>
<tr>
<td><strong>Erythroid (%)</strong></td>
<td>32.1±6.5</td>
<td>25.3±4.8</td>
<td>28.9±6.3</td>
</tr>
<tr>
<td><strong>Granuloids (%)</strong></td>
<td>43.8±2.8</td>
<td>47.3±4.4*</td>
<td>44.2±3.6</td>
</tr>
<tr>
<td><strong>Lymphocytes (%)</strong></td>
<td>18.5±3.9</td>
<td>21.4±1.2</td>
<td>20.4±3.2</td>
</tr>
<tr>
<td><strong>Monocytes (%)</strong></td>
<td>5.2±1.5</td>
<td>4.9±0.8</td>
<td>5.9±2.5</td>
</tr>
<tr>
<td><strong>Megakaryocytes (%)</strong></td>
<td>0.1±0.1</td>
<td>0.2±0.2</td>
<td>0.2±0.2</td>
</tr>
</tbody>
</table>

* p<0.05 VS WT n=4
Table II.

<table>
<thead>
<tr>
<th>counts/section</th>
<th>intima</th>
<th>media</th>
<th>adventitia</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.69±0.23</td>
</tr>
<tr>
<td>apoE-KO</td>
<td>1.00±0.27*</td>
<td>0.00±0.00</td>
<td>1.75±0.39*</td>
</tr>
<tr>
<td>DKO</td>
<td>0.64±0.17*</td>
<td>0.00±0.00</td>
<td>1.57±0.27*</td>
</tr>
</tbody>
</table>

* p<0.05 vs. WT

CD3-positive T-Cell in aorta
Histamine Deficiency Decreases Atherosclerosis and Inflammatory Response in ApoE-KO Mice Independently on Serum Cholesterol Level

Ke-Yong Wang, 1 Akihide Tanimoto,1, 2 Xin Guo, 1 Sohsuke Yamada, 1 Shohei Shimajiri, 1 Yoshitaka Murata, 3 Yan Ding, 1 Masato Tsutsui, 4 Seiya Kato, 5 Teruo Watanabe, 6 Hiroshi Ohtsu, 7 Ken-Ichi Hirano, 8 Kimitoshi Kohno, 9 and Yasuyuki Sasaguri 1*

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Materials and Methods

Measurement of blood pressure

The systolic and diastolic blood pressures were measured by tail-cuff method in 33-week-old mice fed NcD under conscious conditions (Model MK-2000, Muromachi Kikai Co., Ltd., Tokyo, Japan).

Hematopoietic cell counts in peripheral blood and bone marrow

The mice aged 33 wk fed NcD were used for the hematopoietic cell counts. Total white blood cell counts in the peripheral blood were calculated (counts/µL) and the leukocyte fractions (% to total count) were classified into neutrophiles, eosinophiles, basophiles, monocytes and lymphocytes. Total nucleated cells of the bone marrow (femur) were counted (counts/femur) and the fractions (% to total count) were classified into erythroids, granuloids, lymphocytes and megakaryocytes.

Mast cell counts in atherosclerotic lesions

The paraffin sections from the aortic lesions from 12-wk-HcD fed mice were stained by toluidine blue stain and mast cells were counted.

T lymphocyte counts in atherosclerotic lesions

The T lymphocytes in the atherosclerotic lesion from 12-wk-HcD fed mice were detected by immunohistochemistry using anti-CD3 antibody (rabbit polyclonal, x1; Dako) and positive cells were counted per section on a light microscope.

RT-PCR for HH3R and HH4R

The mRNA expression in the atherosclerotic lesions of 12-wk-HcD fed mice was
detected by RT-PCR using the primers listed below. For HH3R mRNA detection, the forward primer was 5’-AGCGCATGAAGATGGTATCC-3’ and the reverse primer was 5’-AGCTTGGTGAAAGGCTCTACG-3’. For HH4R mRNA detection, the forward primer was 5’-ATGGTAGGCAATGCTGTGG-3’ and the reverse primer was 5’-TGTGTTCTGCTGTTCTTC-3’. As an internal control β-actin expression was monitored using the forward (FP) and reverse (RP) primers: FP 5’-TACATGGCTGGGGTGTTGAA-3’ and RP 5’-AAGAGAGGCAATCCTCCACCC-3’.

**Figure Legends**

**Supplemental Fig. I. Body weight and blood pressure in mice fed NcD.**

A) Body weight curve of WT, apoE-KO and DKO mice up to 33-wk old. B) Blood pressure of the mice was measure at the age of 33 wk. Both systolic and diastolic blood pressures were lower in DKO mice than in apoE-KO mice.

**Supplemental Fig. II. HH3R and HH4R expression in atherosclerotic lesions.**

After feeding with HcD for 12 wk, HH3R mRNA but not HH4R expression was increased in apoE-KO mice, which was decreased in DKO mice.

**Supplemental Fig. III. Expression of various inflammatory factors in atherosclerotic lesion.**

After feeding with HcD for 12 wk, mRNA expression was monitored by real time RT-PCR. A) LOX-1  B) IL1R1  C) TNFR2  D) MMP2  E) MMP3  F) iNOS

All the expression was increased in apoE-KO mice than in WT mice and decreased in
DKO mice.