Biochemical Evidence of Atherosclerosis Progression Mediated by Increased Oxidative Stress in Apolipoprotein E–Deficient Spontaneously Hyperlipidemic Mice Exposed to Chronic Cigarette Smoke

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Abstract. Cigarette smoking is a major risk factor for cardiovascular disease. The induction of oxidative stress by smoking plays a key role in the progression of atherosclerosis. However, the underlying mechanisms are not fully understood. In the present study, we investigated whether long-term smoking can accelerate the progression of atherosclerosis and whether oxidative stress is implicated in its pathogenesis. Apolipoprotein E–deficient spontaneously hyperlipidemic mice, a model of atherosclerosis, were exposed to the gas-phase of smoke, from which tar and nicotine had been removed, for 15 min a day, 6 days a week, for 16 weeks. Exposure to cigarette smoke significantly increased the serum levels of oxidative stress markers such as thiobarbituric acid–reactive substances, oxidatively modified low-density lipoproteins, and 3-nitrotyrosine, but it did not affect serum cholesterol and triglyceride levels. Exposure to smoke also accelerated the accumulation of total cholesterol levels in the aorta that was accompanied by an increase in 3-nitrotyrosine levels of the atherosclerotic mice. These changes in the serum and aorta that progressed with exposure to smoke were prevented by vitamin E administration. Our data suggest that chronic cigarette smoking promotes and aggravates atherosclerosis and that the antioxidant vitamin E exerts an anti-atherogenic effect via reduction of oxidative stress.

Keywords: cigarette smoking, atherosclerosis, oxidative stress, apolipoprotein E–deficient spontaneously hyperlipidemic mice, vitamin E

Introduction

Cigarette smoking, either active or passive smoke exposure, has been epidemiologically established as a major risk factor for atherosclerotic vascular disease, including coronary heart disease and stroke (1 – 4). In animal studies, inhalation of cigarette smoke has been reported to promote atherosclerotic plaque development (5, 6). Although the mechanism through which cigarette smoking causes atherosclerosis is unclear, several studies have provided significant evidence for the pathogenesis of atherosclerosis such as increasing the probability of thrombosis (7, 8), raising the systemic inflammation status (9), inducing endothelial dysfunction (10 – 12) and promoting lipid peroxidation (13). The gas phase of cigarette smoke contains many free radicals, oxidant molecules, and pro-oxidant molecules (14, 15), and exposure to cigarette smoke increases systemic oxidative stress in humans (16 – 18) and animals (19). Furthermore, cigarette smoking is thought to increase oxidation of low-density lipoproteins (LDLs) (17, 20). Oxidized LDL plays a key role in the formation of atherosclerotic lesions (21). In addition, circulating oxidized LDL is associated with clinical manifestations of atherosclerosis (22). These findings suggest that oxidative stress may be a pivotal mechanism for the atherogenic effects of cigarette smoking.

Apolipoprotein E (ApoE)-deficient mice develop
severe hypercholesterolemia and atherosclerotic lesions in a manner similar to humans (23, 24), and they are used extensively as a model for atherosclerosis research (25). In these mice, cigarette smoking has been reported to accelerate atherogenesis (26, 27). Catanzaro et al. (28) have suggested that such an accelerating effect of smoking may be due to increased oxidative stress mediated at least in part by the actions of nicotine. In the present study, we investigated the effects of chronic exposure to the gas phase of cigarette smoke, from which tar and nicotine had been removed, on the development of atherosclerosis in ApoE-deficient spontaneously hyperlipidemic mice (29), a new animal model of atherosclerosis, by assessing the changes in various lipid and oxidative stress marker levels in the serum and aorta. We also examined the effect of vitamin E administration on the biochemical parameters affected by the gas phase smoke exposure, as vitamin E is a strong lipophilic antioxidant and protects against free radical peroxidation of LDL.

Materials and Methods

Materials

Chemicals of analytical reagent grade were purchased from Nacalai Tesque (Kyoto) and used without further purification. The cigarettes used were the Frontier Lights brand (JT, Tokyo) containing 1 mg of tar and 0.1 mg of nicotine per cigarette. Cambridge filters (Borgwaldt, Germany) were used to remove 99.9% of all particles and nicotine from the cigarette smoke. ProtEX-DEAE columns (particle diameter: 5 µm, column size: 50 mm × 4.6 mm i.d.), which were used for anion-exchange high-performance liquid chromatography (HPLC), were kindly donated by Mitsubishi Chemical Co. (Tokyo). Water was purified with a Milli Q Jr. (Millipore, Tokyo) and used to prepare eluents for the HPLC method.

Animals

Five-week-old male C57BL/6.KOR/StmSlc-Apoed6 (B6.SHL) mice (29), a line of apoE-deficient spontaneously hyperlipidemic mice with a genetic background of C57BL/6, were purchased from Japan SLC, Inc. (Hamamatsu). All mice were kept in an air-conditioned room (23 ± 2°C and 55 ± 10% humidity) under an artificial 12-h light/dark cycle (7:00 a.m. – 7:00 p.m.). After preliminary breeding for one week, 18 mice were divided equally into three groups (n = 6 each group): the control, smoking, and smoking + VE groups (Table 1). The smoking and smoking + VE groups were exposed to the gas phase of cigarette smoke from which nicotine and tar had been removed by passage through a Cambridge filter, and the control group was exposed to room air. The control and smoking groups were bred with a high-fat diet consisting of 0.15% cholesterol, 14% fat, 46% carbohydrate, and 24% protein (CLEA Rodent Diet Quick Fat supplemented with 0.15% cholesterol; CLEA Japan, Inc., Tokyo). The smoking + VE group was bred with the high-fat diet supplemented with 0.3% vitamin E. Both the diets and water were freely available to the mice for 16 weeks. The body weight was determined at 2-week intervals from 6 weeks of age. Experiments performed on the animals were carried out according to the Guideline Principles for the Care and Use of Laboratory Animals approved by The Japanese Pharmacological Society.

Table 1. Grouping and changes in body weight

<table>
<thead>
<tr>
<th>Group</th>
<th>Diet</th>
<th>Treatment</th>
<th>No. of mice</th>
<th>Body weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>initial</td>
<td>final</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>HC</td>
<td>non-smoking</td>
<td>6</td>
<td>23.2 ± 0.4</td>
</tr>
<tr>
<td>Smoking</td>
<td>HC</td>
<td>smoking</td>
<td>6</td>
<td>23.0 ± 0.6</td>
</tr>
<tr>
<td>Smoking + VE</td>
<td>HC + 0.3% VE</td>
<td>smoking</td>
<td>6</td>
<td>23.1 ± 0.5</td>
</tr>
</tbody>
</table>

HC: a high-fat diet containing 0.15% cholesterol, VE: vitamin E. Data are reported as the mean ± S.E.M.
overnight fasting, at 4-week intervals from 6 weeks of age. The serum was separated by centrifugation (1500 × g for 10 min) from the clotted blood, stored at −80°C and then used for the determination of total cholesterol, triglycerides, thiobarbituric acid–reactive substances (TBARS), oxidatively modified LDL, and glucose. An aliquot of serum was delipidated with ethanol/ether (3:1, v/v) for the determination for 3-nitrotyrosine and 3-chlorotyrosine. At the end of the experiment, mice were killed under ether anesthesia after overnight fasting. The aorta was excised, cleared of adhering fat and connective tissue, and then freeze-dried to constant weight. The lipids in the aorta samples were extracted at 50°C for 20 min with chloroform-methanol (2:1 v/v). After the solvent had been evaporated under nitrogen, the extracts were used for the determination of total and free cholesterol.

The delipidated serum and aorta samples were hydrolyzed with 6 M hydrochloric acid for 24 h at 110°C. After removal of hydrochloric acid from the hydrolysate, the residue was dissolved in distilled water and used for the determination of 3-nitrotyrosine and 3-chlorotyrosine.

**Determination of oxidatively modified LDL**

An anion-exchange HPLC method was used to measure the oxidatively modified LDL in the serum, according to a previously described method (30, 31). Serum LDL was separated by stepwise elution into three subfractions: LDL1, LDL2, and LDL3. The cholesterol level of each subfraction was detected using an enzymatic post-column reaction and evaluated as a relative value to the total cholesterol levels in LDL. The degree of oxidative modification of LDL was assessed by the decrease in LDL1 and the increase in LDL2 and LDL3. Thus, LDL1, LDL2, and LDL3 refer to normal, mildly modified, and severely modified fractions, respectively.

**Determination of 3-nitrotyrosine and 3-chlorotyrosine**

HPLC with electrochemical detection (HPLC-ECD) was used to quantify 3-nitrotyrosine and 3-chlorotyrosine in the serum and aortic proteins, according to a previously described method (31). Briefly, aliquots of serum and aorta samples obtained by the methods described above were injected into the HPLC-ECD system. The voltage of the guard cell was set at 800 mV and those of the first and second electrodes of the analytical cell were set at 550 and at 750 mV, respectively. The analytical column was a Cosmosil 5C18-AR (particle diameter, 5 µm; column size, 150 × 4.6 mm I.D.) equipped with the guard column (10 × 4.6 mm I.D.) described above, and the mobile phase was 20 mM sodium phosphate buffer, pH 3.0, including 5% (v/v) methanol. The flow rate was 1 mL/min and the column temperature was set at 20°C. Quantitation of 3-nitrotyrosine and 3-chlorotyrosine was performed by comparison of the peak areas with those of authentic 3-nitrotyrosine and 3-chlorotyrosine, respectively.

**Biochemical analysis**

Total and free cholesterol levels in the serum and aorta were determined by a fluoro-enzymatic method as described previously (32). Esterified cholesterol concentration was calculated as the difference between total and free cholesterol. Lipid peroxide levels in plasma were estimated by assaying TBARS, using the fluorometric method described by Yagi (33). Triglyceride and glucose levels were evaluated using a Triglyceride E test Wako and Glucose C II test Wako (Wako Pure Chemical Industries, Osaka), respectively. Protein levels were determined using a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA).

**Statistics**

The results are each expressed as the mean ± S.E.M. Statistical analysis (Bonferroni multiple-range test) was performed by using the Stat View software package (SAS Institute Inc., Cary, NC, USA). A difference was considered significant when P<0.05.

**Results**

**Changes in body weight**

Table 1 shows the body weights of B6.SHL mice treated with smoking and vitamin E on the initial and final days of the experiment at 6 and 22 weeks of age, respectively. There were no significant differences in body weight among the groups throughout the experiments, although the body weight of the smoking group tended to decrease after 20 weeks of age.

**Effects of smoking and vitamin E on serum lipid and glucose levels**

Table 2 shows the changes in the serum levels of total cholesterol, triglycerides, and glucose in each group. At 6 weeks of age, B6.SHL mice already displayed extremely high levels of total cholesterol and triglycerides (approximately 7 and 3 times, respectively) in comparison with C57BL/6 mice, the origin of the B6.SHL mice, for which the levels were 106 ± 9 mEq/L and 61 ± 9 mg/dL, respectively. Feeding the mice a high-fat diet containing 0.15% cholesterol caused a marked increase in the total cholesterol levels and a decrease in the triglyceride levels. The glucose levels remained at around 100 mg/dL after 10 weeks of age. However, neither smoking nor vitamin E administration affected these levels throughout the experimental period.
Similarly, these treatments did not affect the cholesterol levels of LDL, very low-density lipoproteins, and high-density lipoproteins (data not shown).

Effects of smoking and vitamin E on serum levels of oxidative stress biomarkers

TBARS, oxidatively modified LDL, 3-nitrotyrosine, and 3-chlorotyrosine were measured as biomarkers of oxidative stress (Fig. 1). The serum levels of TBARS, a marker of lipid peroxidation, significantly increased in the smoking group compared to the control group throughout the experimental period. These levels elevated by smoking were markedly inhibited by vitamin E coadministration with smoking.

The relative levels of LDL subfractions, LDL1, LDL2, and LDL3, were determined as an index of oxidatively modified LDL. By smoking, the levels of LDL1, normal LDL, significantly decreased and those of LDL3, severely modified LDL, significantly increased throughout the experimental period. These changes by smoking were significantly restored by vitamin E coadministration. The levels of LDL2, mildly modified LDL, were not affected by smoking and vitamin E administration throughout the experimental period (data not shown).

3-Nitrotyrosine and 3-chlorotyrosine are specific biomarkers of peroxynitrite (ONOO−) formation and myeloperoxidase (MPO)-catalyzed oxidation, respectively. The serum level of 3-nitrotyrosine significantly increased in the smoking group compared to the control group throughout the experimental period, and the increased level was revived to that of the control group by vitamin E administration. The serum level of 3-chlorotyrosine was not affected by smoking and vitamin E administration throughout the experimental period.

Table 2. Effects of smoking and vitamin E (VE) on total cholesterol, triglyceride, and glucose levels in the serum of apoE-deficient spontaneously hyperlipidemic (B6.SHL) mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Age (weeks)</th>
<th>Total cholesterol (mg/dL)</th>
<th>Triglycerides (mg/dL)</th>
<th>Glucose (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>738 ± 49</td>
<td>211 ± 13</td>
<td>59 ± 4</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>1193 ± 105</td>
<td>112 ± 8</td>
<td>110 ± 4</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>1361 ± 90</td>
<td>81 ± 4</td>
<td>95 ± 3</td>
</tr>
<tr>
<td>Smoking</td>
<td>6</td>
<td>734 ± 56</td>
<td>214 ± 11</td>
<td>59 ± 4</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>1051 ± 79</td>
<td>95 ± 4</td>
<td>109 ± 2</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>1545 ± 67</td>
<td>79 ± 6</td>
<td>102 ± 6</td>
</tr>
<tr>
<td>Smoking + VE</td>
<td>6</td>
<td>739 ± 45</td>
<td>217 ± 11</td>
<td>59 ± 5</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>1138 ± 66</td>
<td>105 ± 6</td>
<td>110 ± 3</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>1300 ± 83</td>
<td>66 ± 5</td>
<td>95 ± 2</td>
</tr>
</tbody>
</table>

Data are reported as the mean ± S.E.M.

Effects of smoking and vitamin E on cholesterol levels in the aorta

Figure 2 shows the total, free, and esterified cholesterol levels in the aorta of each group at the end of the experiment. The levels of total, free and esterified cholesterol in the aorta of the control group were markedly high (2.6, 1.8, and 12.9 times, respectively) in comparison with those of normal mice (34). All these cholesterol levels more significantly increased in the smoking group compared to the control group throughout the experimental period, and these increased levels were significantly revived by vitamin E coadministration. In particular, the variation in the esterified cholesterol level was prominent.

Effects of smoking and vitamin E on 3-nitrotyrosine and 3-chlorotyrosine levels in aorta

Figure 3 shows the 3-nitrotyrosine and 3-chlorotyrosine levels in the aorta of each group at the end of the experiment. Treatment with smoking significantly increased the 3-nitrotyrosine and 3-chlorotyrosine levels 1.7 and 2.1 times, respectively. The increased level of 3-nitrotyrosine was significantly reduced by vitamin E coadministration, but that of 3-chlorotyrosine was unaffected.

Discussion

ApoE-deficient spontaneously hyperlipidemic (B6.SHL) mice develop severe hypercholesterolemic and obvious atherosclerotic lesions (29), as observed in ApoE-knockout mice (35). A high-fat diet leads to further increase in plasma cholesterol, which in turn leads to advancement of atherosclerosis (35, 36). In the present study, we have demonstrated that exposure of B6.SHL mice to the gas phase of cigarette smoke, from
which nicotine and tar had been removed, accelerates accumulation of cholesterol in the aorta together with systemic oxidative stress, and also that vitamin E coadministration effectively alleviates such changes. However, exposure to cigarette smoke did not affect the levels of cholesterol and triglyceride in the serum of the mice. These findings indicate that oxidants in cigarette smoke play a significant role in promoting the atherogenic process because cigarette smoke contains large amounts of pro-oxidants (14, 15).

Oxidative stress and oxidative modification of LDL have been postulated to play a central role in initiation of the atherosclerotic process (37). We established that exposure to cigarette smoke increases the levels of biomarkers of oxidative stress, such as TBARS (end products of lipid peroxidation), oxidatively modified LDL, and 3-nitrotyrosine, in the serum of B6.SHL mice. Therefore, the mice exposed to cigarette smoke are

Fig. 1. Effects of smoking and vitamin E (VE) on thiobarbituric acid–reactive substances (TBARS) (A), oxidatively modified LDL (B), 3-nitrotyrosine (C), and 3-chlorotyrosine (D) levels in the serum of apoE-deficient spontaneously hyperlipidemic (B6.SHL) mice. Oxidatively modified LDL was assessed by the variation in LDL subfractions separated by HPLC: the decrease in LDL1, and the increase in LDL3. Each point represents the mean ± S.E.M. of six mice in each group. *P<0.05: smoking group vs. control group. **P<0.05: smoking + VE group vs. smoking group.

Fig. 2. Effects of smoking and vitamin E on total, free, and esterified cholesterol levels in the aorta of apoE-deficient spontaneously hyperlipidemic (B6.SHL) mice at the end of the experiment. Each column represents the mean ± S.E.M. of six mice in each group. *P<0.05: smoking group vs. control group. **P<0.05: smoking + VE group vs. smoking group.
thought to be in a systemic oxidative stress status. This is supported by evidence that vitamin E could significantly reduce the biomarker levels of oxidative stress developed in the serum of B6.SHL mice exposed to cigarette smoke. 3-Nitrotyrosine is widely recognized to be formed by nitration of protein tyrosine residues with peroxynitrite (ONOO$^-$), which is produced by the rapid reaction of nitric oxide (NO) and superoxide (O$_2^-$). In addition, ONOO$^-$ is a strong oxidizing substance and can induce lipid peroxidation and LDL oxidation (38). It is not known how ONOO$^-$ is formed and involved in nitration of tyrosine residues, although it is possible that ONOO$^-$ included in cigarette smoke is directly absorbed through the pulmonary alveoli into the blood. This active agent could also be generated by activation of inflammatory cells such as neutrophils, monocytes, and tissue-associated macrophages. Myeloperoxidase (MPO), a neutrophil-derived enzyme, catalyzes the formation of hypochloric acid (HOCl), which is a powerful chlorinating oxidant in the presence of hydrogen peroxide and chloride. Thus, 3-chlorotyrosine is considered to be a specific biomarker of MPO-catalyzed and HOCl-mediated protein modification (39). In addition, MPO can also generate 3-nitrotyrosine (40, 41). However, MPO does not seem to contribute to ONOO$^-$ generation, at least systemically, because the serum 3-chlorotyrosine levels in the mice did not change with exposure to cigarette smoke.

Cigarette smoking in humans has been known to raise systemic oxidative stress (16, 17, 42). Increased oxidative stress occurring locally in the vessel wall or systemically appears to be implicated in the pathogenesis of atherosclerosis mediated by cigarette smoke (43). In particular, oxidative modification of the lipid components of LDL is a prominent feature of atherosclerosis (44). However, the precise mechanism for this phenomenon is not fully understood.

A useful quantitative indicator of atherosclerotic involvement of the mouse aorta is measurement of the aortic cholesterol content (45). There is a strong positive correlation between morphological lesions of atherosclerosis and the content of free and esterified cholesterol in the aorta of apoE-deficient mice (46). In the present study, therefore, we assessed atherosclerosis by quantifying the aortic content of cholesterol. As a result, we found that exposure to cigarette smoke accelerates accumulation of total, free, and esterified cholesterol that develops in the aorta of B6.SHL mice. This means that smoking is strongly related to the progression of atherosclerosis. It is supposed that oxidatively modified LDL is taken up by macrophages via scavenger receptors (47), promoting cholesterol ester accumulation, leading to the formation of lipid-laden macrophages (foam cells) and accelerated atherosclerosis (44, 47). This hypothesis is strongly supported by our findings that the aortic cholesterol accumulation increased by smoking is suppressed by administration of the antioxidant vitamin E.

We also found that the aortic levels of 3-nitrotyrosine and 3-chlorotyrosine markedly increased in B6.SHL mice exposed to cigarette smoke. We assume that inflammation and vascular dysfunction develop in the arterial wall by smoking. Chronic smoking has been reported to be associated with endothelial dysfunction (10 – 12). Also, oxidatively modified LDL has been shown to reduce the bioavailability of vascular endo-

![Fig. 3. Effects of smoking and vitamin E on 3-nitrotyrosine (A) and 3-chlorotyrosine (B) levels in the aorta of apoE-deficient spontaneously hyperlipidemic (B6.SHL) mice at the end of the experiment. Each column represents the mean ± S.E.M. of six mice in each group. *$P<0.05$: smoking group vs. control group, $\#P<0.05$: smoking + VE group vs. smoking group.](image-url)
In the present study, we found an increase of 3-chlorotyrosine levels in the aorta but not in the serum. Therefore, MPO seems to develop locally in vessel walls and to be associated with vascular impairment. Vitamin E administration could restore the increased aortic 3-nitrotyrosine levels in B6.SHL mice exposed to cigarette smoke, but did not affect the increased aortic 3-chlorotyrosine levels. Therefore, MPO does not seem to have a strong association with the production of 3-nitrotyrosine in the vessel walls.

In summary, we demonstrated that cigarette smoking greatly accelerates the development of cholesterol accumulation (atherosclerosis) in the aorta of apoE-deficient spontaneously hyperlipidemic (B6.SHL) mice, a model of atherosclerosis. The mechanisms of the accelerated cholesterol accumulation after smoking are complex, but it is certain that locally and systemically increased oxidative stress, particularly oxidation of LDL, is involved in the formation of atherosclerosis because vitamin E coadministration with smoking prevented increases in cholesterol and 3-nitrotyrosine levels in the aorta that accompany an increase of oxidative stress markers in the serum. The present study provides evidence that antioxidant vitamin E supplements can act against the progression of atherosclerosis caused by smoking.

Acknowledgments

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