Farnesyltransferase Inhibitor, Manumycin A, Prevents Atherosclerosis Development and Reduces Oxidative Stress in Apolipoprotein E-Deficient Mice

Michiko Sugita, Hiroki Sugita, Masao Kaneki

Objective—Statins are presumed to exert their antiatherogenic effects in part via lipid-lowering–independent mechanisms. Inhibition of protein farnesylation and/or geranylgeranylation by statins has been postulated to contribute to the lipid-lowering–independent effects. However, a role for protein farnesylation in atherogenesis has not yet been studied. Therefore, we examined the effects of farnesyltransferase inhibitor, manumycin A, on the development of atherosclerosis in apolipoprotein E (apoE)-deficient mice fed a high-fat diet.

Methods and Results—Manumycin A treatment for 22 weeks decreased Ras activity, and reduced fatty streak lesion size at the aortic sinus to 43% of that in vehicle-treated apoE-deficient mice (P<0.05), while plasma total cholesterol was unaltered. Moreover, manumycin A reduced α-smooth muscle actin-positive area to 29% of that in vehicle-treated apoE-deficient mice (P<0.01). The prevention of atherogenesis by manumycin A was accompanied by amelioration of oxidative stress, as judged by reduced ex vivo superoxide production and nitrotyrosine immunoreactivity.

Conclusions—These results indicate that the inhibition of farnesyltransferase prevents the development of mature atherosclerosis with concomitant alleviation of oxidative stress in apoE-deficient mice. The present data highlight farnesyltransferase as a potential molecular target for preventive and/or therapeutic intervention against atherosclerosis.

Key Words: apoE-deficient mice ■ atherosclerosis ■ farnesyltransferase ■ oxidative stress ■ Ras

3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) have had a major impact by decreasing cardiovascular events in humans.1,2 The efficacy of statins has been considered to be primarily explained by their lipid-lowering property. However, a growing body of evidence highlights the lipid-lowering–independent effects of statins.3,4 Rapid onset of clinical benefits and weak correlations between plasma cholesterol levels and coronary lumen change or cardiovascular events indicate an involvement of nonlipid-lowering actions of statins.5–7 In fact, statins exhibited the beneficial effects to increase flow-mediated vasodilation in normcholesterolemic subjects,8–10 as well. Recently, the National Cholesterol Education Project (NCEP) Adult Treatment Panel III guideline recommended that patients with diabetes and cardiovascular disease should initiate statin therapy regardless of baseline low-density lipoprotein cholesterol levels,11 although more evidence is needed to support a universal recommendation of statin therapy for all patients with diabetes and without cardiovascular disease. In animal models of atherosclerosis, lipid-lowering–independent beneficial effects of statins have been shown. Low doses of statins reduce atherogenesis without altering cholesterol level in rabbits fed high-cholesterol diet,12–15 apolipoprotein E (apoE)*3-leiden transgenic mice,16 and allograft atherosclerosis.17

The nonlipid-lowering effects of statins are presumed to be accounted for by direct pleiotropic actions on the vessel wall, which include anti-inflammatory and antioxidant effects of the drugs.18–20 Nevertheless, the molecular mechanisms by which statins exert these pleiotropic actions remain to be determined.

3-Hydroxy-3-methylglutaryl-coenzyme A reductase is the rate-limiting enzyme of cholesterol synthesis. The inhibition of this enzyme results in decreased production of not only cholesterol but also geranyl pyrophosphate and farnesyl pyrophosphate, leading in turn to reduced protein isoprenylation, namely, geranylgeranylation and farnesylation, respectively. Of note, statins inhibit DNA replication and cell cycle progression in many cell types, including vascular smooth muscle cells, independent of the inhibition of cholesterol synthesis. The inhibitory effects of statins on cell proliferation were rescued by the addition of precursors of protein isoprenylation, farnesol, or geranylgeraniol, but not by cholesterol.21 These previous findings suggest that the inhibition of farnesylation or geranylgeranylation may be important for
cholesterol-independent pleiotropic effects of statins. Thus, statins have been proposed to exert their nonlipid-lowering effects through the inhibition of isoprenylation.4

Farnesylation is critical for activation of Ras family small G-proteins. A role of Rho family small G-proteins including Rac in atherogenesis has been characterized.23 Rac is activated by geranylgeranylation, but not by farnesylation. However, a role for Ras family small G-proteins or farnesyltransferase in the pathogenesis of atherosclerosis has not been extensively studied. Therefore, to investigate a role for farnesyltransferase in the development of atherosclerosis, we examined the effects of the farnesyltransferase inhibitor, manumycin A, in apoE-deficient mice fed a high-fat diet.

Materials and Methods

Animals

Male apoE-deficient mice on C57BL/6 background and male wild-type C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, Me). The Institutional Animal Care Committee approved the study protocol. The apoE-deficient and wild-type mice were housed in mesh cages in a room maintained at 25°C and illuminated in 12:12-hour light–dark cycles; they were provided with a high-fat diet and water ad libitum. The high-fat diet contained 58.0% calories from fat, 25.6% from carbohydrates, and 16.4% from proteins (D12309; Research Diets, New Brunswick, NJ).

Treatment With Manumycin A

The treatment with farnesyltransferase inhibitor, manumycin A (5 mg/kg body weight dissolved in 0.4% dimethyl sulfoxide, phosphate-buffered saline, subcutaneously 3 times per week; Sigma, St. Louis, Mo), or vehicle alone was commenced at 7 weeks of age in apoE-deficient mice fed a high-fat diet. The treatment with manumycin A was continued for 22 weeks. Then, after overnight fasting, blood samples were obtained to measure plasma cholesterol with a commercial kit (Sigma).

Analysis of Fatty Streak Lesion and Smooth Muscle-Like Cells in Neointima

After the 22-week treatment, fatty streak lesion at the aortic sinus was evaluated in apoE-deficient mice. The α-smooth muscle actin expression was examined by immunohistochemistry and immunoblotting. For further details, see the supplemental information (available at http://atvb.ahajournals.org).

Detection of Superoxide Generation

Superoxide generation was evaluated by dihydroethidium labeling. For further details, see the supplemental information.

Detection of Tyrosine-Nitrated Proteins

Tyrosine-nitrated proteins were detected by immunohistochemistry23 and immunoblotting. For further details, see the supplemental information.

Determination of Activation Status of Ras

The aorta of apoE-deficient and age-matched wild-type C57BL/6 mice was homogenized as described previously.24 The abundance of Ras, Raf-1, and phosphorylated Raf-1 was determined by immunoblot analysis with anti-Pan-Ras (EMD Biosciences, San Diego, Calif), Raf-1 (Upstate, Lake Placid, NY), and phosphorylated Raf-1 (Cell Signaling, Beverly, Mass). Immunoblotting was performed as previously described.24 For a more detailed description of determination of the activation status of Ras, particularly evaluation of active GTP-bound Ras and farnesylated Ras, see the supplemental information.

![Figure 1. Manumycin A treatment reduced active Ras, farnesylated Ras, and phosphorylation of Raf-1 in apoE-deficient mice. Immunoblotting (IB) revealed that active GTP-bound Ras (A), farnesylated Ras (C), and phosphorylated Raf-1 (D) were elevated in the aorta of vehicle-treated apoE-deficient mice relative to wild-type (WT) mice, and that manumycin A treatment decreased them. However, the protein expression of Ras (B) and Raf-1 (E) was unaltered by apoE deficiency or manumycin A. C indicates vehicle; M, manumycin A. *P<0.05; **P<0.01.](image)

Statistical Analysis

The data were compared using 1-way ANOVA followed by Fisher protected least significant difference test. A value of P<0.05 was considered statistically significant. All values were expressed as means±SEM.

Results

Manumycin A Treatment Decreased Activation of Ras Pathway in the Aorta of ApoE-Deficient Mice

The treatment with farnesyltransferase inhibitor, manumycin A (5 mg/kg body weight, subcutaneously, 3 times per week), for 22 weeks did not affect plasma total cholesterol level (360±34.7, 399±46.8 mg/dL in vehicle- and manumycin A-treated animals, respectively), food intake (2.76±0.20, 2.53±0.05 g/d in vehicle- and manumycin A-treated animals, respectively), and body weight gain (vehicle: 23.0±0.5, 32.9±1.2, 33.0±1.4 g; and manumycin A: 23.2±0.4, 32.4±1.2, 33.2±2.0 at 0, 10 and 22 weeks after inception of the treatment, respectively).

To examine the effects of manumycin A on the activation status of Ras in the aorta of apoE-deficient mice, we evaluated active GTP-bound Ras, farnesylated Ras, and phosphorylation (activation) of Raf-1, an immediate downstream signaling molecule of Ras. Active Ras, farnesylated Ras, and phosphorylated Raf-1 were significantly increased in the aorta of vehicle-treated apoE-deficient mice compared with wild-type mice. Manumycin A treatment reverted increased active Ras, farnesylated Ras, and phosphorylated Raf-1 in apoE-deficient mice (Figure 1A, IC, ID). The abundance of total Ras and Raf-1 proteins did not differ between wild-type mice, and manumycin A- and vehicle-treated apoE-deficient mice (Figure 1B, 1E). These results clearly indicate that manumycin A decreased the activity of the Ras pathway in apoE-deficient mice, which was elevated relative to wild-type mice.
Manumycin A Treatment Reduced Fatty Streak Lesion Size in ApoE-Deficient Mice

We examined the effects of manumycin A on fatty streak lesion at the aortic sinus, a hallmark for early development of atherosclerosis. In vehicle-treated apoE-deficient mice, robust oil red O-positive lesion was observed at the aortic sinus. However, the fatty streak lesion size in manumycin A-treated apoE-deficient mice was significantly reduced to 43% of that observed in vehicle-treated apoE-deficient mice (P<0.05; Figure 2). In the aorta of wild-type mice, neither fatty streak lesion nor neointima was observed (data not shown).

Manumycin A Reduced Vascular Smooth Muscle-Like Cells in the Neointima in ApoE-Deficient Mice

Next, we examined the effects of farnesyltransferase inhibitor on the increase in vascular smooth muscle-like cells in neointima, a pathognomonic feature of the progression of mature atherosclerosis. In vehicle-treated apoE-deficient mice, α-smooth muscle actin-positive area was prominent in the neointima as well as in the media. However, α-smooth muscle actin-positive area in the neointima in manumycin A-treated apoE-deficient mice was diminished in size to 29% of that observed in vehicle-treated apoE-deficient mice (P<0.01; Figure 3A, 3B). In contrast, the extent of α-smooth muscle actin immunoreactivity was similar in the media of both manumycin A- and vehicle-treated animals. Immunoblot analysis also demonstrated reduced expression of α-smooth muscle actin by manumycin A, as compared with vehicle (Figure 3C), while β-actin expression was unaltered (Figure 3D).

Manumycin A Ameliorated Oxidative Stress in the Aorta of ApoE-Deficient Mice

The prevention of atherosclerosis development by manumycin A was accompanied by the amelioration of oxidative stress in apoE-deficient mice. In vehicle-treated apoE-deficient mice, substantial ex vivo superoxide generation was observed in the neointima. The incubation with Mn (II) TMPyP, a cell-permeable superoxide dismutase-mimetic, abolished ex vivo superoxide-derived signal, indicating the specificity of detection of superoxide. Manumycin A treatment significantly reduced ex vivo superoxide generation as compared with vehicle-treated apoE-deficient mice (Figure 4). The mitigation of oxidative stress by manumycin A was corroborated by immunohistochemical analysis with anti-nitrotyrosine antibody. In manumycin A-treated apoE-deficient mice, the immunoreactivity for nitrotyrosine, a surrogate marker for oxidative stress, was significantly reduced compared with vehicle-treated apoE-deficient mice (Figure 5A). Decreased tyrosine nitration by manumycin A was further confirmed by immunoblot analysis (Figure 5B).

Figure 2. Manumycin A treatment reduced fatty streak lesion size in the aorta of apoE-deficient mice. Treatment with manumycin A resulted in a significant reduction in oil red O-positive area at the aortic sinus of apoE-deficient mice fed a high-fat diet.

Figure 3. Manumycin A treatment reduced smooth muscle-like cells in the aorta of apoE-deficient mice. A, Treatment with manumycin A resulted in a significant reduction in α-smooth muscle actin-positive area in the neointima of apoE-deficient mice. M, I, and L indicate media, intima, and lumen, respectively. B and C, Immunoblot analysis (IB) also revealed reduced α-smooth muscle actin (SM-α-actin) in manumycin A-treated apoE-deficient mice as compared with vehicle. β-actin expression was unaltered by manumycin A.

Figure 4. Manumycin A treatment suppressed superoxide generation in the aorta of apoE-deficient mice. In situ generation of superoxide was evaluated using dihydroethidium ex vivo. In the aorta of apoE-deficient mice treated with manumycin A, superoxide generation was decreased, as compared with apoE-deficient mice treated with vehicle alone. Cell-permeable superoxide dismutase-mimetic, Mn (II) TMPyP, abolished the signal, indicating the specificity of the assay.
Nitrotyrosine to aminotyrosine,26 and prevented the immuno-
expression did not differ between the groups. Incubation with
altering plasma cholesterol level (Figures 2 and 3). The
aorta of apoE-deficient mice fed a high-fat diet. The reduction of farnesylated Ras, active Ras, and phosphorylated Raf-1 by manumycin A (Figure 1) indicates that manumycin A treatment effectively reduced farnesylation and activation of the Ras pathway, as expected. Consistent with previous studies,27,28 oxidative stress and activation of the Ras pathway were elevated in vehicle-treated apoE-deficient mice relative to wild-type mice.

Previous studies have shown that pharmacological inhibition of farnesyltransferase by manumycin A,29 or FFT-277,30 or transfection of dominant-negative mutant of this enzyme31 inhibits proliferation, migration, and superoxide production in cultured vascular smooth muscle cells. Previous studies showed that the gene transfer of dominant negative mutant of Ras to vasculature suppressed intimal thickness induced by carotid artery injury in vivo.32,33 Taken together, the direct effects of manumycin A in vasculature is assumed to contribute to the protective effects of the farnesyltransferase inhibitor in the aorta of apoE-deficient mice.

A body of work in animal models and in cultured cells indicates an important contributory role for oxidative stress in atherogenesis.34–36 Antioxidants attenuated fatty streak lesion in apoE-deficient mice34,35 and hypercholesterolemic rabbits. Reactive oxygen species are involved in activation of macrophages, and proliferation, migration, and dedifferentiation of vascular smooth muscle cells.37,38 Therefore, it is reasonably conceivable that the amelioration of oxidative stress by manumycin A may contribute to the reduction of fatty streak lesion and smooth muscle-like cell accumulation in the neointima.

Farnesyltransferase inhibitors have been shown to exert cytostatic or proapoptotic effects in cultured cells, and the clinical trials are underway to evaluate its efficacy to treat the patients with cancer or leukemia.39 Inhibition of the Ras pathway is considered to play an important role in the anti-cancer activity of farnesyltransferase inhibitors. Of interest, NAD(P)H oxidase-mediated superoxide production is required for oncogenic Ras-induced transformation,40 and farnesyltransferase inhibitors reduce reactive oxygen species generation in transformed cells. Likewise, farnesyltransferase inhibitor FTI-227 blocked NAD(P)H oxidase-mediated superoxide generation induced by IL-1β, platelet-derived growth factor, or constitutively active mutant H-Ras in vascular smooth muscle cells.30 In contrast, Rac1, a major regulator of NAD(P)H oxidase activity, is not a direct target of farnesyltransferase inhibitor, because Rac1 is activated by geranylgeranylation, but not by farnesylation.41 It is reasonably conceivable, therefore, that decreased activity of the Ras pathway by manumycin A may mediate the amelioration of oxidative stress in the aorta of apoE-deficient mice.

However, oxidative stress also causes activation of the Ras pathway in various cell types including vascular smooth muscle cells.42–43 Hence, it is possible that reduced oxidative stress may also contribute to decreased activation of the Ras

Tyrosine-nitrated proteins were increased in the aorta of vehicle-treated apoE-deficient mice compared with wild-type mice. Manumycin A treatment reduced tyrosine-nitrated proteins in apoE-deficient mice, although manumycin A did not fully reverse it to the level in wild-type mice. However, β-actin expression did not differ between the groups. Incubation with sodium dithionite (100 mmol/L) for 1 hour, which reduces nitrotyrosine to aminotyrosine,26 and prevented the immunostaining and immunoblotting with anti-nitrotyrosine antibody (supplemental Figures I and II, available online at http://atvb.ahajournals.org), indicating the specificity of the detection of nitrotyrosine.

Discussion
We found that the treatment with farnesyltransferase inhibitor, manumycin A, reduced fatty streak lesion size and the area of vascular smooth muscle-like cells in the neointima in the aorta of apoE-deficient mice fed a high-fat diet without altering plasma cholesterol level (Figures 2 and 3). The prevention of the development of atherosclerosis was accompanied by the amelioration of oxidative stress in apoE-deficient mice (Figures 4 and 5). These data indicate that the inhibition of farnesyltransferase prevents accumulation of lipid-laden macrophages and proliferation and/or migration of smooth muscle-like cells in the neointima, and also reduces oxidative stress in the aorta of apoE-deficient mice fed a high-fat diet. The reduction of farnesylated Ras, active Ras, and phosphorylated Raf-1 by manumycin A (Figure 1) indicates that manumycin A treatment effectively reduced farnesylation and activation of the Ras pathway, as expected. Consistent with previous studies,27,28 oxidative stress and activation of the Ras pathway were elevated in vehicle-treated apoE-deficient mice relative to wild-type mice.

Figure 5. Manumycin A treatment reduced nitrotyrosine immuno-
activity in the aorta of apoE-deficient mice. A, Immunohisto-
chemical analysis revealed that immunoreactivity for nitroty-
rosine was decreased in the aorta of apoE-deficient mice

treated with manumycin A compared with those treated with
vehicle alone. Normal IgG served as a negative control. Arrow
denotes the internal elastic lamina. B, Immunoblotting also dem-
strated that tyrosine-nitrated proteins were increased in the
aorta of vehicle-treated apoE-deficient mice compared with WT mice, and that elevated tyrosine nitration was decreased by
manumycin A treatment in apoE-deficient mice. However, β-
actin expression did not differ between the groups. *P<0.05;
**P<0.01 vs wild-type, §§P<0.01 vs vehicle.

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pathway in manumycin A-treated apoE-deficient mice. Based on the reciprocal relationship between these 2 signaling cascades, one can reasonably speculate that positive feedback loop between oxidative stress and activation of the Ras pathway may be formed in the disease conditions associated with atherogenesis, and that farnesyltransferase inhibition might prevent atherogenesis by blocking this vicious cycle.

Our data seem to be in accord with a previous study by George et al., showing that a selective inhibitor for Ras, farnesyl thiosalicylic acid, attenuated fatty streak lesion in apoE-deficient mice. However, the differences also appear to exist in the effects of farnesyl thiosalicylic acid and manumycin A. In the study of George et al., whereas 6-week treatment with this agent was associated with marked attenuation (52% reduction) in fatty streak lesion size, the effects of 10-week treatment with farnesyl thiosalicylic acid was less pronounced (28% reduction). These data seem to indicate that functional inhibition of Ras by farnesyl thiosalicylic acid might be more effective on early atherogenesis compared with mature atherosclerosis. In contrast, we found that 22-week treatment with farnesyltransferase inhibitor, manumycin A, reduced mature atherosclerosis, resulting in 57% and 71% reduction in fatty streak lesion size and α-smooth muscle actin-positive area, respectively. Although the major substrates of farnesyltransferase are Ras family small G-proteins, other proteins such as RhoB, nuclear lamins, and some protein tyrosine phosphatases are also the targets for farnesyltransferase. Thus, the apparent difference in the effects of Ras inhibitor, farnesyl thiosalicylic acid, and farnesyltransferase inhibitor, manumycin A, could be explained by the effects of manumycin A on other substrates of farnesyltransferase than Ras. However, further studies will be required to clarify this point.

Because dimethyl sulfoxide is a hydroxyl radical scavenger, we cannot exclude the possibility that the injection of dimethyl sulfoxide itself might exert a beneficial effect. However, our preliminary observation revealed that administration of vehicle containing dimethyl sulfoxide by itself did not affect fatty streak lesion size at the aortic sinus in apoE-deficient mice on a high-fat diet (unpublished observation, M. Kaneki, 2005). It is important to note that the same dose of dimethyl sulfoxide was administered to both manumycin A- and vehicle-treated animals.

A potential contributory role of infection, particularly, that of Chlamydia pneumoniae, in atherosclerosis has been suggested. One can speculate, therefore, that antibiotic property of manumycin A might also contribute to the beneficial effects of manumycin A. However, previous studies and our observations do not appear to support this possibility. In apoE-deficient mice, it remains controversial whether C. pneumoniae accelerates atherogenesis. Antibiotics failed to ameliorate atherosclerosis in apoE-deficient mice. Moreover, antibacterial and antifungal activities of manumycin A are modest, although manumycin A is a potent farnesyltransferase inhibitor. We found that manumycin A did reduce farnesylated Ras (Figure 1C). Collectively, it seems unlikely that the protective effects of manumycin A might be substantially attributed to antibiotic activity rather than inhibition of farnesyltransferase.

In summary, farnesyltransferase inhibitor, manumycin A, significantly inhibited the development of mature atherosclerosis and reduced oxidative stress in the aorta of apoE-deficient mice. The present study suggests the possibility that inhibition of farnesylation may be involved in lipid-lowering–independent beneficial effects of statins. However, it remains to be clarified whether the concentrations of statins in human vasculature are really high enough to inhibit farnesylation in situ. The present data highlight farnesyltransferase as a potential molecular target to prevent the progression of atherosclerosis.

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Disclosures
None.

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Supplemental Information

*Farnesyltransferase Inhibitor, Manumycin A, Prevents Atherosclerosis Development and Reduces Oxidative Stress in ApoE-Deficient Mice*

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Methods

**Analysis of Fatty Streak Lesion and Smooth Muscle-like Cells in Neointima**

Fatty streak lesion at the aortic sinus was evaluated as previously described (Ref. 1) with minor modifications. At 29 weeks of age, male apoE-deficient mice treated with manumycin A (n=10) or vehicle (n=8) for 22 weeks, and male age-matched wild-type mice (n=6) were anesthetized with pentobarbital sodium (50 mg/kg BW, i.p.). The body weight did not differ between wild-type (WT) mice, and manumycin A- and vehicle-treated apoE-deficient mice at 29 weeks of age (WT: 33.5 ± 1.4 g; manumycin A: 33.2 ± 2.0; vehicle: 33.0 ± 1.4). After the aorta was perfused with PBS, the heart was embedded in OCT compound (Tissue-Tek, Electron Microscopy Sciences, Hatfield, PA) and serial cross sections 10 µm thick were cut by cryostat. Serial sections at intervals of 50 µm were prepared, stained with oil red O and counterstained with hematoxylin.
Cryosections were also stained with α-smooth muscle actin antibody (DAKO, Carpinteria, CA) to visualize smooth muscle-like cells. The area positive for oil red O or anti-α-smooth muscle actin was measured by using NIH image 1.62 software (NTIS, Springfield, VA), and the average of 4-5 sections was taken as a representative value for each animal. The protein expression of α-smooth muscle actin in the aorta was also evaluated by immunoblotting.

**Detection of Superoxide Generation *ex vivo***

Superoxide generation was evaluated as previously described (Ref. 2). In brief, frozen, enzymatically intact, 10 μm-thick sections of the aorta were incubated with dihydroethidium (0.3 μM in PBS, Molecular Probes, Eugene, OR) for 30 min at 37°C in a humidified chamber protected from light. On reaction with superoxide, dihydroethidium is oxidized to the fluorescent molecule, ethidium bromide, which binds to DNA in the nucleus. Ethidium bromide-derived fluorescence was visualized with LSM Pascal laser scanning confocal microscope (Carl Zeiss, Thornwood, NY) using a 543 nm He-Ne laser combined with a 560 nm-long pass filter. To confirm the specificity of superoxide detection, dihydroethidium was incubated with cell-permeable, superoxide dismutase-mimetic, Mn (II) TMPyP (50 μM, Cayman Chemical, Ann Arbor, MI) in the indicated experiments.

**Determination of Activation Status of Ras**

The aorta of apoE-deficient mice were homogenized in homogenization buffer (50 mM HEPES/NaOH, pH 7.5, 2 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride
[PMSF], 10 mM sodium pyrophosphate, 5 µg/ml aprotinin, and 5 µg/ml leupeptin, 1 mM dithiothreitol). The abundance of Ras, Raf-1 and phosphorylated Raf-1 was determined by immunoblot analysis with anti-Pan-Ras (EMD Biosciences, San Diego, CA), Raf-1 (Upstate, Charlottesville, VA) and phosphorylated Raf-1 (Cell Signaling, Beverly, MA) antibodies.

Active, GTP-bound Ras was assessed as previously described (Ref. 3). In brief, the homogenates were adjusted to 0.3% NP-40, and incubated in the presence of 10 mM MgCl₂ with recombinant protein of Ras binding domain of Raf-1 that was fused to glutathione-S-transferase (Upstate). The absorbates to glutathione agarose were subjected to immunoblotting with anti-Pan Ras antibody.

Farnesylated Ras was evaluated by the Triton X-114 partition method as previously described (Ref. 4) with minor modifications. In brief, the aorta was homogenized in lysis buffer (50 mM HEPES/NaOH, pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 1 mM PMSF, 1 mM dithiothreitol, 1 mM sodium vanadate, 1 mM sodium pyrophosphate, 1% Triton-X-100, 0.5% SDS, 10 µg/mg aprotinin, 10 µg/mg leupeptin).

Equal volumes of lysate and 4% Triton X-114 were combined in a borosilicate glass tube, vortexed, and incubated. The detergent phase of the solution was subjected to immunoblotting with anti-Pan Ras antibody.

**Immunohistochemistry for Nitrotyrosine**

Tissue was fixed in 4% paraformaldehyde for 10 min at 4°C. The slides were incubated with blocking buffer (3% bovine serum albumin [BSA], 0.1% TritonX-100/PBS) for 30 min at room temperature. Then the sections were incubated with anti-nitrotyrosine
antibody (Upstate) or normal rabbit IgG diluted at 1: 50 in 3% BSA, 0.1% TritonX-100/PBS for 1 h at room temperature. After washing, the sections were incubated in a solution of 1:200 of Alexa Fluor 488 conjugated goat anti-rabbit IgG antibody (Molecular Probes) for 1 h at room temperature. The slides were then washed, mounted, viewed with a fluorescence microscope and photographed with a Spot RT digital camera.

**Detection of Tyrosine-nitrated Proteins by Immunoblotting**

The homogenates of the aorta, which were incubated with or without sodium dithionite (100 mM) for 1 h at room temperature, were separated by SDS-PAGE, and equal amounts of proteins were transferred to nitrocellulose membranes. The membranes were incubated with or without anti-nitrotyrosine antibody (Cell Signaling) at 1:1,000 dilution at 4°C overnight, then incubated with horseradish peroxidase conjugated anti-rabbit IgG antibody at 1:50,000 dilution at 4°C for 2 h. The antigen-antibody complexes were visualized with an enhanced chemiluminescence kit (Amersham). Immunoblots were scanned using a scanner (HP Scanjet 4850, Hewlett-Packard, Palo Alto, CA) and immunoreactive proteins were quantified by using NIH image 1.62 software.
References to the Supplemental Information


Figure Legends

Supplemental Figure I. Specificity of nitrotyrosine immunostaining at the aortic sinus of apoE-deficient mice. After fixation, slides were preincubated with or without 100 mM sodium dithionite for 1 h, which reduces nitrotyrosine to aminotyrosine. Preincubation with sodium dithionite prevented immunostaining for nitrotyrosine in the aorta from vehicle-treated apoE-deficient mice, indicating the specificity of detection of nitrotyrosine.

Supplemental Figure II. Specificity of immunoblot analysis for tyrosine-nitrated proteins. A, Immunoblot analysis with anti-nitrotyrosine antibody demonstrated increased tyrosine-nitrated proteins in the aorta of vehicle-treated apoE-deficient mice relative to wild-type (WT) mice, and that treatment with manumycin A decreased tyrosine-nitrated proteins in the aorta of apoE-deficient mice. Lanes 7, 8 and 9 are identical to the lanes 1, 2 and 3 in Figure 5. The equal amount of proteins from the same animal (vehicle-treated apoE-deficient mice) was loaded into lanes 8 and 10 after incubation without and with sodium dithionite (100 mM) for 1 h, respectively. In vitro incubation with sodium dithionite almost completely abolished the immunoreactivity (lane 10). B, When the incubation with anti-nitrotyrosine antibody (primary antibody) was omitted, otherwise the same procedure of the incubation with secondary antibody (horseradish peroxidase-conjugated anti-rabbit IgG antibody) alone did not result in significant signals. These results did confirm the specificity of the immunoblot analysis
for tyrosine-nitrated proteins.  W: wild-type; C: vehicle; M: manumycin A; D: sodium dithionite.
Dithionite

Nitrotyrosine

(−)

(+)
Supplemental Figure II (Sugita)

A

IB: Nitrotyrosine

B

Secondary Antibody Only

kDa

30 35 50 75 105 160 250

kDa

30 35 50 75 105 160 250