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Long-term effects of resveratrol supplementation on suppression of atherogenic lesion formation and cholesterol synthesis in apo E-deficient mice

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ABSTRACT

Atherosclerosis is a chronic inflammatory disease of the arteries resulting from interactions between lipids, monocytes, and arterial wall cells. The effects of resveratrol supplements (RV, 0.02% and 0.06% each, w/w) with regard to the modulation of lipid profiles, cholesterol synthesis, and anti-atherogenesis were examined in apo E-deficient (apo $E^{-/-}$) mice fed a normal diet. The concentration of total-cholesterol (total-C) and LDL-cholesterol (LDL-C) in plasma was significantly lower in the resveratrol-supplemented groups compare to the control group over the entire experimental period. The plasma HDL-C concentration was significantly elevated, and the ratio of HDL-C/total-C was significantly higher in the CF and RV groups than in the control group. Plasma paraoxonase (PON) activity was significantly higher in the 0.06% resveratrol group. The hepatic HMG-CoA reductase (HMGR) activity was significantly lower in the clofibrate and resveratrol groups than in the control group. Resveratrol supplements attenuated the presence of atherosclerotic lesions and periarterial fat deposition in the apo $E^{-/-}$ mice. The presence of intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) in atherosclerotic vessels was diminished in the resveratrol-supplemented apo $E^{-/-}$ mice. These results provide new insight into the anti-atherogenic and hypocholesterolemic properties of resveratrol in apo $E^{-/-}$ mice that were fed a normal diet.

Increased plasma cholesterol is known to be a major risk factor related to the development of atherosclerosis [1]. Excessive amounts of cholesterol in the blood can destroy membrane function or result in atherosclerotic damage [2]. Differentiation of monocytes to macrophages and internalization of lipids by macrophages, forming foam cells, result in the development of fatty streak lesions [3]. The recruitment of monocytes is regulated by endothelial adhesion molecules and their corresponding monocyte ligands [4]. For example, ICAM-1 has been reported to be upregulated in the endothelium of human atherosclerotic plaques [5]. The expression of VCAM-1, which supports monocyte adhesion to cytokine-treated endothelial cells, is also rapidly induced on the aortic endothelium of rabbits fed an atherogenic diet [6].

The intake of flavonoids led to an inverse relation with mortality due to coronary heart disease (CHD) and the incidence of myocardial infarction in the Zutphen Elderly Study [7]. A high intake of flavonoids, at approximately 30 mg/day, was associated with a reduction in the CHD mortality rate compared with individuals having a low flavonoids intake [7]. Trans-resveratrol (3,4′,5-trihydroxystilbene), a naturally occurring phytoalexin primarily found in grapes and other plants, was implicated as the main active principle agent [8]. The effects of resveratrol in biological systems are wide-ranging, as it can act as an apoptotic factor or an anti-inflammatory [9] or anti-oxidant agent [10]. Also, resveratrol has been proven to exhibit cardioprotective [11] and neuroprotective [12] effects. One of stillbenes, resveratrol is found in low quantities in red wine, ranging from 0.3 to 7 mg aglycones/L. Since resveratrol is found in such small quantities in a normal diet, any protective effect is unlikely to be observed at levels occurring normal nutritional intake [13].

Among apolipoproteins involved in atherosclerosis or hypercholesterolemia, apo E is a component of lipoprotein remnants and serves as a ligand in receptor mediated lipoprotein uptake by the liver [14]. The apo $E^{-/-}$ mouse serves as a good model for human atherosclerosis because it mimics the formation and progression of human atherogenic lesions [15]. Apo $E^{-/-}$ mice exhibit their plasma cholesterol levels at 400–500 mg/dL, even on a normal diet, mainly due to the accumulation of VLDL remnants and develop severe atherosclerotic lesions throughout the arterial tree [16].

The current study investigated the overall effects of resveratrol on plasma lipid profile, cholesterol synthesis, and aortic fatty plaque formation in apo $E^{-/-}$ mice fed a normal diet.

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Materials and methods

Animals and diets. Four-week-old male apo $E^{-/-}$ mice (weighing 20–22 g) were purchased from the Jackson Laboratories (Bar Harbor, ME). After allowing a week for adaptation, all mice were randomly divided into four groups. The mice were fed an AIN-76 semisynthetic diet that was supplemented with 0.02% (w/w) clofibrate (CF, Sigma Chemical Co.), 0.02% (w/w) resveratrol (RV, Sigma Chemical Co.) or 0.06% (w/w) resveratrol for 20 weeks. Blood was periodically taken from the inferior vena cava for determination of the plasma lipids during the animal experiment and at the end of the experimental period, respectively. Livers were removed, rinsed with physiological saline, and weighed for enzyme analysis and lipid measurement. All samples were stored at −70 °C until analysis. The current study protocol was approved by the Ethics Committee at Kyungpook National University for animal studies.

Lipid and collagen analyses. Plasma lipid concentrations were determined by using enzymatic kits (Sigma Diagnostics, Chemical Co., St. Louis, MO). The hepatic tissues were homogenized in a 20 mM potassium phosphate buffer (pH 7.4) and hepatic lipids were extracted using chloroform and methanol (1:1, v/v) solution. Triton X-100 and a sodium cholate solution were added to the dissolved lipid sample for emulsification. The hepatic cholesterol and triglycerides were analyzed with the same enzymatic kit as used in the plasma analyses. Collagen concentration in the liver was determined using a Sircol Collagen assay kit (Biocolor Newtownabbey, UK).

Hepatic 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR) and Acyl-CoA:cholesterol acyltransferase (ACAT) activities. Hepatic microsomes were prepared according to the method of Hulcher and Oleson [17], with some slight modifications. Two grams of liver tissues were homogenized in 4 ml of ice-cold buffer (pH 7.0) containing 0.1 M triethanolamine, 0.02 M EDTA and 2 mM dithiothreitol (DTT; pH 7.0). The homogenates were centrifuged twice, at both 10,000g and 12,000g, for 15 min at 4 °C. The supernatants were ultra-centrifuged twice at 100,000g for 60 min at 4 °C. The resulting microsomal pellets were redissolved in 1 ml of homogenization buffer without DTT, and the microsomal protein concentrations were determined using the Bradford method [18] and analyzed for HMGR and ACAT activities. The microsomal HMGR activities were measured with [^{14}C]-HMG-CoA as the substrate, based on a modification of the method of Shapiro et al. [19]. The microsomal ACAT activities were determined using [^{14}C]-oleoyl CoA according to the method of Gillies et al. [20].

Histopathological analyses of atherosclerotic lesions. Each aortic arch was removed and wrapped with saline-soaked guaze after removing the connective tissues. All were fixed in 10% paraformaldehyde/PBS, embedded in paraffin, and then stained with hematoxylin and eosin (H&E). Another section of the aortic arch was cryosectioned and stained with Oil-Red O solution. For immunohistochemistry, the aortic arch was cryostat sectioned, fixed in hydrogen peroxide, and washed in citrate buffer (pH 6.0). These sections were treated with blocking reagent (Ultra Tech HRP, USA) to prevent nonspecific binding, and incubated with monoclonal antibodies against VCAM-1 or ICAM-1 (SantaCruz Biotech., Inc.). Antibody reactivity was detected by using HRP-conjugated biotin–streptavidin complexes and developed with diaminobenzidine tetrahydrochloride as the substrate.

Lipid peroxidation levels and paraoxonase activity. Plasma and erythrocyte samples were mixed with 5% trichloroacetic acid (TCA) and 60 mmol/L thiobarbituric acid (TBA). After incubation at 80 °C for 90 min, the supernatants were centrifuged at 1000g for 15 min at 4 °C, and the absorbance recorded at 535 nm by using tetramethoxypropane (Sigma Chemical Co.) as the standard. Hepatic homogenates containing 8.1% sodium dodecyl sulfate (SDS) and distilled water were mixed with 20% acetic acid (pH 3.5) and 0.8% aqueous TBA solution, and subsequently heated at 95 °C for 60 min. After cooling, *n*-butanol and pyridine (15:1, v/v) solutions were added and the samples were centrifuged. The absorbance of upper layer was measured at 535 nm. PON activities were spectrophotometrically assayed using plasma and hepatic microsomes. The assay mixture consisted of 1 mM paraoxon in 0.1 M Tris–HCl buffer (pH 8.0) containing 2 mM $CaCl_2$. The increase in absorbance was monitored photometrically for 90 s at 405 nm and 25 °C.

Statistical analysis. The parameter values were all expressed as the means ± standard error. Significant differences among the groups were determined by one-way ANOVA analysis using the SPSS program (SPSS Inc., Chicago, IL). The differences between the means were assessed using Duncan's multiple-range test, and statistical significance was considered at $p < 0.05$.

Results

Plasma lipids

Initial total-C concentration in plasma exhibited approximately the same values in all four groups. However, the plasma total-C level was reached its peak at the 6th week and gradually decreased by the 20th week (Table 1). The concentration of plasma triglycerides also reached the highest level at the 6th week and then was significantly reduced by 30%, 17%, and 18%, in the 0.02% CF, 0.02% RV, and 0.06% RV groups, respectively, as compared to the control group. Also, the LDL-C concentration was significantly lowered by 25%, 41%, and 27% by the 0.02% CF, 0.02% RV, and 0.06% RV supplements (Table 2), respectively. The plasma HDL-C concentration was significantly elevated, and the ratio of HDL-C/total-C was significantly higher in the CF and RV groups than in the control group. For these reasons, the atherogenic index (AI) was significantly lower in the 0.02% CF, 0.02% RV, and 0.06% RV groups than in the control group, by 34%, 44%, and 46%, respectively. The apo-AI/apo B ratio was also significantly higher in the 0.02% and 0.06% RV groups compared to the control group.

Hepatic HMG-CoA reductase/ACAT activities and lipid profiles

The HMG-CoA reductase activity was significantly lowered in the 0.02% CF, 0.02% RV, and 0.06% RV supplement groups compared

Table 1
Effects of resveratrol supplementation for 20 weeks on the age-dependent changes in the concentration of plasma lipids in apo $E^{-/-}$ mice fed a normal diet

	C[d]	CF-0.02%[e]	RV-0.02%[f]	RV-0.06%[g]
Total-cholesterol (mmol/L)				
0 week	6.59 ± 0.38	6.53 ± 0.30	6.48 ± 0.40	6.33 ± 0.42
6 week	29.63 ± 1.59[a]	16.07 ± 0.80[b]	24.23 ± 1.53[c]	23.30 ± 3.02[c]
18 week	13.39 ± 1.04[a]	10.99 ± 0.57[ab]	10.02 ± 0.92[b]	12.18 ± 0.76[ab]
20 week	9.60 ± 0.50[a]	7.73 ± 0.36[b]	6.33 ± 0.41[c]	7.78 ± 0.51[b]
Triglycerides (mmol/L)				
0 week	0.76 ± 0.03	0.8 ± 0.03	0.83 ± 0.04	0.77 ± 0.04
6 week	2.61 ± 0.05[a]	1.83 ± 0.17[b]	2.17 ± 0.29[b]	2.13 ± 0.09[b]
14 week	2.13 ± 0.25[a]	1.23 ± 0.07[b]	0.97 ± 0.08[b]	1.43 ± 0.15[b]
20 week	1.51 ± 0.17[a]	1.10 ± 0.11[ab]	1.00 ± 0.11[b]	1.42 ± 0.16[ab]

Data are means ± SE values of 10 mice per group.
[abc]Means in the same row not sharing a common superscript are significantly different among groups at $p < 0.05$.
[d] C, control diet.
[e] CF-0.02, 0.02% clofibrate-supplemented diet.
[f] RV-0.02, 0.02% resveratrol-supplemented diet.
[g] RV-0.06, 0.06% resveratrol-supplemented diet.

Table 2
Effects of resveratrol supplementation for 20 weeks on plasma and hepatic lipids profiles in apo $E^{-/-}$ mice fed a normal diet

	C[d]	CF-0.02%[e]	RV-0.02%[f]	RV-0.06%[g]
Plasma (mmol/L)				
LDL-C[h]	8.1 ± 0.5[a]	6.1 ± 0.4[b]	4.8 ± 0.3[c]	5.9 ± 0.5[bc]
HDL-C[i]	1.07 ± 0.10[a]	1.41 ± 0.12[b]	1.36 ± 0.11[b]	1.87 ± 0.12[c]
HDL-C/total-C (%)	11.7 ± 0.9[a]	18.4 ± 1.5[b]	20.9 ± 1.7[b]	22.3 ± 1.5[b]
AI[j]	7.0 ± 0.5[a]	4.6 ± 0.4[b]	3.9 ± 0.4[b]	3.8 ± 0.3[b]
Apo[k]-AI/apo B ratio	0.34 ± 0.05[a]	0.40 ± 0.06[ab]	0.67 ± 0.09[b]	0.51 ± 0.06[b]
Liver (mg/g liver)				
Triglycerides	79.43 ± 6.57	88.16 ± 4.55	78.08 ± 4.06	74.17 ± 8.10
Cholesterol	3.03 ± 0.07[a]	3.15 ± 0.08[a]	2.68 ± 0.05[b]	2.36 ± 0.12[b]
HMGR[l] (pmoles/min/mg protein)	282.15 ± 15.62[a]	148.20 ± 31.38[b]	113.73 ± 6.20[b]	152.97 ± 11.45[b]
ACAT[m] (pmoles/min/mg protein)	49.99 ± 4.17	52.81 ± 2.86	55.92 ± 2.78	59.77 ± 3.43
Collagen	97.8 ± 1.6	94.7 ± 3.9	88.1 ± 1.7	95.5 ± 9.1
Apo-AI/apo B ratio	0.39 ± 0.07	0.48 ± 0.03	0.59 ± 0.16	0.80 ± 0.18

Data are means ± SE values of 10 mice per group.
[abc]Means in the same row not sharing a common superscript are significantly different among groups at $p < 0.05$.
[d] C, control diet.
[e] CF-0.02, 0.02% clofibrate-supplemented diet.
[f] RV-0.02, 0.02% resveratrol-supplemented diet.
[g] RV-0.06, 0.06% resveratrol-supplemented diet.
[h] LDL-C, Low-density lipoprotein cholesterol.
[i] HDL-C, high-density lipoprotein.
[j] AI, atherogenic index, (TC-HDL-cholesterol)/HDL-C.
[k] Apo, apolipoprotein.
[l] HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase.
[m] ACAT, acyl-CoA cholesterol acyltransferase.

to the control group (Table. 2). However, there was no significant difference in the hepatic ACAT activity among the groups.

The hepatic cholesterol content was significantly lowered in the two resveratrol-supplemented groups as compared to the control group (Table 2). However, the two different levels of dietary resveratrol resulted in no significant changes in the hepatic triglycerides and collagen contents.

Histopathological analyses of atherosclerotic lesions

Pathological evaluations of arterial lesions and lipid depositions were also carried out in this study. The histopathological changes of the aortic arch dissection were prominent in the ascending aorta, as seen after staining with hematoxylin and eosin (Fig. 1A), as well as Oil-Red O (Fig. 1B). A fatty plaque was found at the edge of the dissection that extended over the inside of the aortic arch in the control group of apo $E^{-/-}$ mice. Known as macrophage lipids, these lesions were stained by Oil-Red O, which allows for visualization of lipid accumulation in lesions. The lesions were exposed to granulation including internalization of lipids, but their formation was prevented by the inclusion of clofibrate and resveratrol supplements. The sections of aortic arch taken from control mice revealed advanced atherosclerotic lesions, containing both foam cells and cholesterol clefts. However those taken from resveratrol-supplemented mice showed no visible intimal lesions. ICAM-1 and VCAM-1 stained sections of the aortic arch were dissected from similar anatomic locations from each group. The aortas from the control group contained a fatty plaque that was readily visible upon ICAM-1 exposure (Fig. 1C). Histological examination also confirmed the presence of abundant VCAM-1 within the fatty plaque of the control group (Fig. 1D).

Lipid peroxidation levels and paraoxonase activity

Plasma TBARS levels were significantly lower in the 0.06% RV group, and erythrocyte TBARS levels were found to be lower in the 0.02% and 0.06% RV groups. Hepatic TBARS levels were significantly lower in the 0.02% CF and 0.06% RV-supplemented groups than in the control group (Table 3). Plasma PON activity was significantly higher only in the 0.06% RV group than in the control group. In contrast, hepatic PON activity was significantly lowered by 0.02% CF supplement.

Discussion

Plasma LDL-cholesterol is an important risk factor regarding the development and progression of atherosclerosis. The reduction of plasma total-C and LDL-C levels plays a major role in mediating the regression of atherosclerosis. Apo $E^{-/-}$ mice were used to investigate the supplementary effects of resveratrol in regards to the suppression of atherogenesis when a normal diet was provided. Fatty streaks can be generally developed as early as 8 weeks in apo $E^{-/-}$ mice, and after 15 weeks, advanced lesions consist of a fibrous cap covering a necrotic core with numerous foamy macrophages have been observed [21].

In the present study, the major effect of resveratrol appears to be the stimulation of an increase in the apo-AI/apo B ratio and levels of HDL-cholesterol, as well as a decrease in plasma LDL-C concentration and hepatic HMG-CoA reductase activity. An elevation of HDL-C and apo-AI levels improves HDL function, which in turn either prevents aortic lesion formation or causes the regression of existing aortic lesions in apo $E^{-/-}$ mice [22]. The severity of atherosclerosis is positively correlated with the concentration of circulating triglyceride-rich lipoproteins, and negatively related to apo-AI and HDL levels [23]. Interestingly, the major changes resulting from two different doses of resveratrol appeared to be a decrease in the total-C, LDL-C, and triglycerides concentration and an increase in HDL-C concentration, apo-AI/apo B ratio, and PON activity in the plasma. Decreased serum PON activity was found in patients with familial hypercholesterolemia, which is associated with accelerated atherogenesis [24]. Moreover, recent large-scale clinical trials revealed that the PON enzyme associated with HDL-C protects both LDL-C and HDL-C against oxidation [25]. Although PON is inactivated by lipid peroxides, it hydrolyzes and reduces lipid peroxides and cholesteryl linoleate hydroperoxides in both oxidized lipoproteins and atherosclerotic lesions [26]. Interestingly, a 0.06% RV supplement only elevated the plasma PON activity with a simultaneous decrease in the hepatic TBARS

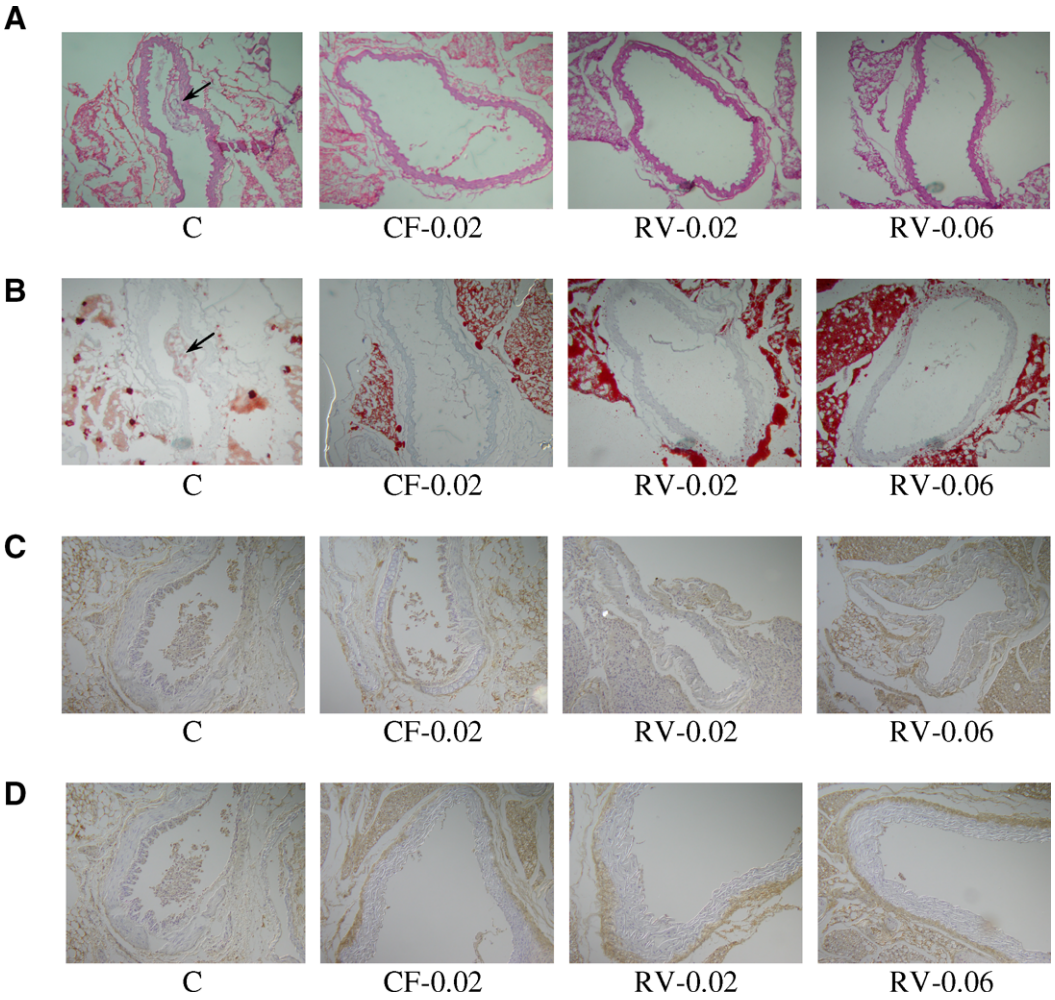

Fig. 1. A histological observation of aortas from apo $E^{-/-}$ mice supplemented with clofibrate or resveratrol. (A) H&E stained transverse-section of aortic arch; (B) Oil-Red O stained cryosection of aortic arch; (C) Immunostained transverse-section of aortic arch with anti-ICAM-1; (D) Immunostained transverse-section of aortic arch with anti-VCAM-1. Advanced fatty plaques containing lipid-rich components were present in the aortic arch, as well as cholesterol crystal deposition in the control group of apo $E^{-/-}$ mice fed a normal diet (arrows: fatty streak. Magnification 200×). C, control diet; CF-0.02, 0.02% clofibrate-supplemented diet; RV-0.02, 0.02% resveratrol-supplemented diet; RV-0.06, 0.06% resveratrol-supplemented diet.

Table 3
Effects of resveratrol supplementation for 20 weeks on TBARS levels and paraoxonase activity in apo $E^{-/-}$ mice fed a normal diet

	C[d]	CF-0.02%[e]	RV-0.02%[f]	RV-0.06%[g]
TBARS[h]				
Plasma (nmol/ml/min)	7.43 ± 2.03[ab]	8.58 ± 0.53[ab]	10.40 ± 2.40[a]	5.30 ± 0.58[b]
Erythrocyte (nmol/ml/min)	4.13 ± 0.15[ab]	4.42 ± 0.22[a]	3.58 ± 0.10[b]	3.79 ± 0.15[b]
Liver (nmol/mg pro/min)	42.84 ± 4.43[a]	28.33 ± 3.33[bc]	35.98 ± 4.47[ac]	18.13 ± 2.06[b]
Paraoxonase				
Plasma (nmol/ml/min)	64.61 ± 13.13[a]	92.02 ± 11.58[ab]	77.06 ± 14.77[ab]	112.41 ± 19.11[b]
Liver (nmol/mg pro/min)	4.12 ± 0.78[a]	1.08 ± 0.31[b]	3.40 ± 0.16[a]	4.25 ± 0.68[a]

Data are means ± SE values of 10 mice per group.
[abc]Means in the same row not sharing a common superscript are significantly different among groups at $p < 0.05$.
[d] C, control diet.
[e] CF-0.02, 0.02% clofibrate-supplemented diet.
[f] RV-0.02, 0.02% resveratrol-supplemented diet.
[g] RV-0.06, 0.06% resveratrol-supplemented diet.
[h] TBARS, thiobarbituric acid substances.

level, but no changes were observed in the plasma and erythrocyte TBARS concentrations. In general, serum PON activity is negatively correlated with serum TBARS concentration, whereas HDL levels are positively correlated with PON activity [27].

Two doses of dietary resveratrol, 0.02% and 0.06%, seemed to inhibit hepatic HMG-CoA reductase, which may reduce the hepatic cholesterol pool and thus prevent the cholesterol accumulation in the liver. Another possible action of resveratrol is an increase in cholesterol uptake by hepatic LDL receptors, which may accelerate the decrease in the concentration of plasma cholesterol in resveratrol-supplemented apo $E^{-/-}$ mice. The inhibition of HMG-CoA reductase by the resveratrol supplement could possibly be considered as a new cholesterol-lowering approach, thereby reducing the risk of developing atherosclerosis. In addition, resveratrol can

decrease tissue collagen contents and slow aortic lesion development. In the current study, resveratrol supplementation was given to apo $E^{-/-}$ mice prior to development of atherosclerosis and as a result inhibited the progression of fatty streak formation as compared to the control group. Resveratrol supplements significantly suppressed the formation of fatty plaques and the elevation of plasma cholesterol concentrations, with a simultaneous increase in the apo-AI/apo B ratio. However, there seemed to be a species difference in the anti-atherogenic property of resveratrol. Previous studies showed that red wine polyphenols can prevent the development of atherosclerosis in both apo $E^{-/-}$ mice [28] and hamsters [29], whereas dietary resveratrol did not lower the plasma cholesterol levels in diet-induced hypercholesterolemic rabbits [30]. When trans-resveratrol was injected daily into female rats at 20 and 40 mg per kg body weight, their lipoprotein profile remained unaffected after 21 days of treatment, and there was no change in the level of peroxidation of plasma lipids [31].

Important events during the early steps of atherosclerotic lesion formation include the recruitment of blood monocytes to the vascular wall. Animal models regarding atherosclerosis have demonstrated that monocyte attachment to the arterial vascular endothelium appears to precede the development of fatty streak lesions [32]. The level of VCAM-1 was also elevated prior to leukocyte recruitment in cholesterol-induced lesion formation in mice [5]. This is consistent with our result regarding the appearance of VCAM-1 and ICAM-1 in the ascending arteries in apo $E^{-/-}$ mice.

Resveratrol supplementation improved most of the proatherogenic variables determined in this study, including the levels of VCAM-1 and ICAM-1, when compared to the control group, thereby delaying the progression of atherosclerosis. During the advanced stages of atherosclerosis, the lesions become mature and more resistant to drug or nutrient interventions. However, this study shows the resveratrol supplement can exert its beneficial effect in the prevention of atherosclerosis when provided prior to the development of fatty streaks.

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