The angiotensin-converting enzyme inhibitor, fosinopril, and the angiotensin II receptor antagonist, losartan, inhibit LDL oxidation and attenuate atherosclerosis independent of lowering blood pressure in apolipoprotein E deficient mice

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Abstract

Objective: To investigate the possible mechanisms of the antiatherosclerotic effects of the angiotensin-converting enzyme (ACE) inhibitor, fosinopril, in apolipoprotein (apo) E deficient mice. Methods: Apo E deficient (E\(^\text{\textsuperscript{\text{-}}}\)) mice at the age of 8 weeks received either placebo or a high dose (25 mg/kg/d) of fosinopril supplemented in their drinking water. Results: After 12 weeks of treatment, fosinopril reduced the aortic lesion size by 70%, compared with the placebo group. At this dosage, fosinopril significantly reduced blood pressure from 93±2 mmHg before treatment to 70±2 mmHg at the end of the treatment period (\(P<0.005\)). Fosinopril also increased the resistance of the mice plasma low density lipoprotein (LDL) to CuSO\(_4\)-induced oxidation, as shown by a 90% reduction in the LDL content of malondialdehyde (MDA) and also by a prolongation of the lag time required for the initiation of LDL oxidation (from 100 min in the placebo-treated mice to more than 240 min in the fosinopril-treated mice; \(P<0.001\)). In addition, fosinopril inhibited CuSO\(_4\)-induced oxidation of LDL that was obtained from the aortas of the treated mice, as shown by an 18% and 37% reduction in the LDL content of lipid peroxides and hydroperoxy-cholesterol linoleate, respectively, compared with the placebo-treated mice (\(P<0.01\)). A low dosage of fosinopril (5 mg/kg/d) that was still adequate to reduce their plasma ACE activity and LDL propensity to lipid peroxidation was insufficient to lower their blood pressure. This dosage also reduced the aortic lesion size in the apo E deficient mice by 40% (\(P<0.01\)). Conclusions: The antiatherogenic effects of fosinopril in apo E deficient mice are due not only to blood pressure reduction but also to the direct inhibition of angiotensin II-dependent effects, which are probably also associated with the inhibition of LDL oxidation. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Atherosclerosis; Apo E deficient mice; LDL oxidation; Angiotensin II; Blood pressure

1. Introduction

Angiotensin II (Ang II) is a powerful growth stimulant that can lead to increased cardiac hypertrophy and smooth muscle hyperplasia in the vascular wall [1–5]. Angiotensin-converting enzyme (ACE) inhibitors act as vasodilators, but the most obvious potential benefit is their effect on the renin–angiotensin–aldosterone system by reducing the levels of Ang II [1–5]. Clinical studies have demonstrated that ACE inhibitors significantly reduce the morbidity and mortality of patients with myocardial infarction or heart failure [1–5]. They also reduce the incidence of recurrent myocardial infarction and ischemic events in patients with coronary artery disease [6,7]. Data from a variety of animal experiments indicate that ACE inhibitors can attenuate the development of atherosclerosis across a wide
range of species, including Watanabe heritable hyperlipidemic rabbits, cholesterol-fed Cynomolgus monkeys and minipigs [8–12]. The mechanisms by which ACE inhibitors affect atherosclerosis are not well understood, but it has been postulated that these agents may have multiple effects, including blood pressure lowering [8,9], an antiproliferative effect on vascular smooth muscle cells [3], inhibiting platelet aggregation [13], decrement of plasminogen activator inhibitor-1 (PAI-1) [14] and the attenuation of LDL oxidation [15,16].

Studies in humans and in animal models have shown that oxidative modification of LDL plays a crucial role in the build-up of the atherosclerotic lesions, leading to coronary artery disease [17–23]. We have previously shown that Ang II stimulates macrophage lipid peroxidation, and by so doing, can lead to enhanced cell-mediated oxidation of LDL, resulting in the formation of atherogenic oxidized LDL [24,25]. We have also shown that LDL derived from hypertensive patients is more susceptible to lipid peroxidation than LDL derived from normotensive controls [26] and that ACE inhibitors significantly attenuate the propensity of LDL to oxidation [15].

Recently, we demonstrated that the ACE inhibitor, captopril, which contains a sulfhydryl group, attenuated the progression of atherosclerosis in E0 mice and this effect was associated with a significant inhibition of plasma LDL oxidation [16]. In that study, the contribution of blood pressure-lowering on the attenuation of atherosclerosis was not assessed.

Several studies have shown that the antiatherosclerotic effect of ACE inhibitors could be dissociated from their blood pressure-lowering ability [12,27,28]. On the other hand, it was recently shown that in hypertensive rats, a high dosage of the ACE inhibitor ramipril increased the lifespan more than a low dose which was without an antihypertensive effect [29]. Thus, the contribution of blood pressure-lowering to the antiatherogenic effect of the ACE inhibitors is still controversial.

The benefits of ACE inhibitors have been attributed to blockade of Ang II production. In recent years, an alternative therapeutic method using Ang II receptor antagonists has been investigated. A non-peptide Ang II receptor antagonist, such as losartan, can selectively block the Ang II type I (AT1) receptor without increasing bradykinin levels [30], which is responsible for some of the side-effects of ACE inhibitors (e.g. angioneurotic edema and cough). Furthermore, since Ang II may be produced by alternative pathways [31,32], such drugs may have additional advantage over ACE inhibitors where blockade of the effect of Ang II is incomplete. Thus, blocking the actions of Ang II at the receptor level represents an attractive approach for therapy. Recently, we have demonstrated that a high dose of losartan (25 mg/kg/d) attenuated the progression of atherosclerosis in apo E deficient mice and this was associated with a significant inhibition of plasma LDL oxidation [33].

Apo E deficient mice are suitable for such studies since they develop severe hypercholesterolemia on a low fat, low cholesterol chow diet [34,35], and extensive atherosclerosis with lesions progressing from lipid-laden fatty streaks to advanced fibroproliferative lesions by age 20–30 weeks [36,37]. We have shown previously [22] that in these mice accelerated atherosclerosis is associated with increased lipid peroxidation.

Thus, the aim of this study was to evaluate the respective roles of blood pressure reduction, LDL oxidation inhibition and ACE inhibition on the attenuation of atherosclerosis by fosinopril or losartan in apo E deficient mice.

2. Methods

The ACE inhibitor, fosinopril, was kindly provided by Bristol-Myers Squibb (BMS, Princeton, New Jersey, USA). The angiotensin II receptor antagonist losartan was kindly provided by Dr. Ronald Smith, Merck, Sharp and Dohme (MSD, Whitehouse Station, New Jersey, USA). Apo E deficient mice were created by gene targeting in mouse embryonic stem (ES) cells as previously described [34,35]. In these mice, apo E deficiency causes severe hypercholesterolemia on chow diet, primarily due to elevated levels of very low and intermediate density lipoprotein.

2.1. Experiment 1: High dose of fosinopril

At 8 weeks of age, apo E deficient mice were assigned randomly to placebo or to high dose fosinopril groups. Treatment consisted of 25 mg/kg/d of fosinopril as a supplement in the drinking water. Mice (20 in each group) were supplemented with the drug for 12 weeks.

2.2. Experiment 2: Low dosages of fosinopril or losartan

In order to examine the contribution of blood pressure lowering on the antiatherosclerotic effect of the ACE inhibitor fosinopril and the Ang II receptor antagonist losartan, E0 mice at the age of 4 weeks were assigned to four groups, ten in each. Treatment consisted of 5 mg/kg/d of fosinopril, 5 mg/kg/d of losartan (these doses were found to have no significant antihypertensive effects), hydralazine, a vasodilator that causes direct relaxation of vascular smooth muscles, at a dosage of 5 mg/kg/d (which was found to exert similar hypotensive effects as 25 mg/kg/d of fosinopril), and placebo.

At the end of the treatment period, blood was collected in all groups of mice from the retroorbital plexus under anesthesia with ether [22] into Eppendorf tubes with 1 mmol/l Na,EDTA. Plasma cholesterol was determined enzymatically [22] and serum ACE activity was assayed.
according to Holmquist et al. [38]. This method measures the reduction in the absorbance at 340 nm which results from ACE-induced hydrolysis of furulcroylophenylglycemic (FAPGG) to furulcroyloheptamylalanine (FAP) and glycylglycine.

LDL (d=1.006–1.063 g/ml) was isolated from 3 ml of pooled plasma from each group, by sequential density ultracentrifugation as previously described [22]. The LDL protein content was determined by the method of Lowry et al. [39].

2.2.1. LDL oxidation

LDL was dialyzed overnight against phosphate buffered saline (PBS) to remove the EDTA before oxidation. LDL derived from the different groups was diluted with EDTA-free PBS to a final concentration of 100 mg of protein per ml and then incubated in the presence or absence of 10 mM CuSO₄ at 37°C for 3 h. At the end of the incubation, the oxidative state of plasma LDL was assayed by determination of the thiobarbituric acid reactive substance (TBARS) assay [40]. In addition, the lag time and the kinetics of LDL oxidation were determined by monitoring the formation of conjugated dienes after the supplementation of freshly prepared CuSO₄ (10 mmol/l) by monitoring the increase in absorbance at 234 nm, observed at room temperature (23 °C) every 10 min for a period of 3 h [41].

2.2.2. Blood pressure

Blood pressure measurements were performed, using a tail cuff, before and at the end of the treatment period in all animals [42]. Computerized blood pressure measuring was performed using the IITC model 31 (IITC/Life Science Instruments, USA). Animals were put in a special holder for mice several minutes before beginning the test (to prevent temperature stress, the holder was preheated to 36°C before placing the animals in the holder). The mouse tail was placed into a device with rubber cuff and photoelectric sensor. The blood pressure was measured using special computer software (Blood Pressure Software, IITC, Model 31). The results were displayed as data plots and the summary of digital values (systolic, diastolic and mean blood pressure) was shown on a computer screen (Intel Pentium 100). All measurements were repeated four times for each animal.

2.2.3. Analysis of aortic atherosclerotic lesions

At the end of the experimental period, mice were sacrificed, ten from the high dose fosinopril and ten from the placebo groups. The heart and entire aorta were rapidly dissected out and fixed in 3% glutaraldehyde in 0.1 mol/l sodium cacodylate buffer (pH=7.4) with 0.01% calcium chloride at room temperature. Since it was shown that the aortic origin region with the valves and bifurcation is the most susceptible for atherosclerosis [34–37], we restricted our study to the aortic arch for comparative histomorphometric studies of atherosclerotic lesion development. Thus, the aortic arch was dissected free from the surrounding fatty tissue and the first 4 mm of the ascending aorta (beginning with the aortic valves) removed and cut transversely with razor blades into four blocks of ≈1 mm each. The samples were kept in the fixative overnight prior to rinsing and storage in 0.1 mol/l sodium cacodylate buffer containing 7.5% wt/vol. sucrose. This step was followed by treatment with 1% aqueous solution of osmium tetroxide for 4 h, cacodylate rinse, dehydration in ethanol and propylene oxide, and embedding in epoxy resin (Eponate 12, Pelco). Transverse sections (1 μm) were cut for light microscopy. The prolonged osmium treatment stains the intraluminal, intramural, and intracellular lipid a dense black color. Osmium staining ‘en bloc’ is an excellent method for lipids and, without further staining, shows up the atherosclerotic lesions in thin epoxy-embedded sections at much higher resolution than oil red O staining in frozen sections [16]. Moreover, the sections can be cut on the ultramicrotome and stained with alkaline toluidine blue to provide even better resolution [16].

‘Atherosclerotic lesion’ was defined as the area of (abnormal) pathological structure change. Lesional areas were determined by using a computerized quantitative image-analysis system (Cue-2, Olympus) with appropriate morphometric software. The imaging system consists of a Zeiss Universal R photomicroscope (×10 objective) fitted with a Panasonic WV-CD50 video camera and 14-inch Sony color monitor and IBM-compatible PC. Image analysis was performed on aortic arches from placebo-treated mice and mice treated with hydralazine, fosinopril and losartan. Approximately 80 transverse sections (0.05 mm separation) were taken in total from each animal, and of those, sections with atherosclerotic lesions were selected and marked for image analysis.

In the present study, standardized ‘windows’ (fields of measurement) with an area of 176 758 μm² were used as follows: placebo mice, hydralazine, fosinopril and losartan. The results were pooled for each group and presented as average cross-sectional lesion area per experimental group.

In order to study the oxidizability of LDL derived from the aortic lesions, mice treated with high dose fosinopril or placebo were sacrificed (another ten from each group), aortas dissected, placed in cold (4°C) PBS and processed on the same day. Aortic tissues were rinsed several times in cold PBS. Lipoproteins were gently extracted as previously described [16,33]. One gram of wet tissue (ten mice) was placed in tubes containing 5 ml of 150 mM NaCl, 0.01 M phosphate buffer, pH=7.4 (‘extraction buffer’) and incubated overnight at 4°C, with gentle agitation. To prevent in vitro LDL modification, the following compounds were added to the extraction buffer: 0.01% sodium azide, 0.05% glutathione, 0.08% benzamidine and 0.01% Na₂EDTA. At the end of incubation, the medium supernatant was collected after centrifugation at 10 000×g for 15 min. This supernatant was further centrifuged in a SW41
rotor at 40,000×g for 30 min at 10°C. The pellet was discarded and the rest of the solution was used for density gradient ultracentrifugation. The density of this supernatant was adjusted to 1.250 g/ml by the addition of KBr. A 6-ml sample was placed in a tube and overlaid with 6 ml of lipoprotein extraction buffer (d=1.0 g/ml). After 48 h of centrifugation at 4°C, using SW41 rotor, four visualized bands were separated. These lipoprotein bands were located by their light scattering under indirect illumination, and LDL fractions (d=1.020–1.050 g/ml) were collected by gentle pipetting. A 50-μl lipid extract of lesioned LDL in acetone was analyzed by reversed-phase HPLC using an RP-8 column (4×25 mm, 5-μm particle size, Merck). Methanol/H₂O (97:3, v/v) was used as the mobile phase and the elute was analyzed by UV–VIS detector (Varian IC Star 9050) at 234 nm. Retention time of cholesteryl linoleate hydroperoxide was 29.6 min [43].

2.2.4. Statistical analysis
Results are expressed as the mean ±SD or mean±SEM. ANOVA was used to analyze the significance of the results. Statistical significance was defined as P<0.05.

3. Results

3.1. Effect of a high dose of fosinopril on blood pressure, LDL oxidation and on the progression of atherosclerosis in apo E deficient mice

Apo E deficient mice at the age of 8 weeks were treated with a high dose of fosinopril (25 mg/kg/d) for a period of 12 weeks.

3.1.1. Plasma ACE activity and cholesterol levels
Plasma ACE activity was 768±28 μl/l in the placebo-treated group and was substantially reduced by fosinopril to 180±91 μl/l. Plasma cholesterol in the placebo-treated E₀ mice was 893±56 mg/dl and was not significantly affected by the administration of fosinopril (906±34 mg/dl).

3.1.2. Blood pressure and atherosclerosis
At a dose of 25 mg/kg/d, fosinopril reduced the mean blood pressure from 93±2 mmHg before treatment to 70±2 mmHg at the end of the treatment period (P<0.005). In the placebo group, blood pressure was 89±3 mmHg before treatment and did not change significantly at the end of the treatment period (91±3 mmHg) (Fig. 1A).

Fosinopril administration for 12 weeks led to a significant decrease in the development of atherosclerotic lesions and the average lesion area was significantly lower in the fosinopril-treated group (24,500±2,200 μm²), in comparison with the placebo-treated mice (78,060±5600 μm²) (P<0.001) (Figs. 1B, 2).

3.1.3. LDL oxidation
After 12 weeks of treatment, the susceptibility of the mice LDL to lipid peroxidation following its incubation with 10 μM CuSO₄ was significantly reduced in the apo E deficient mice that were treated with fosinopril compared with the placebo-treated group, as shown by a 90% reduction in the LDL MDA content (P<0.001) (Fig. 3A).

Kinetic analysis of LDL oxidation was also performed by continuously monitoring the formation of LDL conjugated dienes at 234 nm after the addition of 10 μM CuSO₄ to the LDL (100 μg of protein per ml) that was derived from the different treated groups. After 12 weeks of treatment, the lag time required for the initiation of CuSO₄-induced LDL oxidation was 100 min for LDL derived from the placebo-treated group. In contrast, in the fosinopril-treated group, no LDL oxidation was observed even after 240 min of incubation (P<0.001) (Fig. 3B).

It is suggested that LDL oxidation takes place mainly in
the arterial wall. Thus, we also analyzed the effect of fosinopril on the basal oxidative state of LDL-like particles that were isolated from the mice aortic lesions. The content of total peroxides in these LDL-like particles was 17% lower in the fosinopril-treated mice in comparison with LDL-like particles from the aortic lesions of placebo treated mice ($P<0.01$) (49.4±2 and 59.8±5 nmol/mg of LDL protein, respectively). Furthermore, we demonstrated a 37% lower LDL-like particle content of cholesteryl linoleate hydroperoxides in the fosinopril-treated group compared with the placebo group ($P<0.01$) (23.5±2 and 36.9±3 nmol/mg of LDL protein, respectively).

### 3.2. Effect of low dosages of fosinopril or losartan on blood pressure, LDL oxidation and on the progression of atherosclerosis in apo E deficient mice

Apo E deficient mice at the age of 4 weeks were treated with a low dose of fosinopril (5 mg/kg/d), a low dose of losartan (5 mg/kg/d) or with hydralazine (5 mg/kg/d) for 10 weeks.

#### 3.2.1. ACE activity

Plasma ACE activity was 750±38 µ/l in the placebo-treated mice and was still substantially reduced by a low dose of fosinopril to 220±81 µ/l (Table 1), but was not affected by a low dose of losartan (790±28 µ/l), or hydralazine (780±36 µ/l).

#### 3.2.2. Blood pressure and atherosclerosis

In the placebo-, fosinopril- and losartan-treated groups, mean blood pressure levels before treatment were 91±3 mmHg, 89±4 mmHg and 90±3 mmHg, respectively, and did not change significantly at the end of the treatment period (Fig. 4A). As an added control, we used hydralazine at a dose of 5 mg/kg/d which reduced blood pressure from 92±4 mmHg before treatment to 78±4 mmHg at the end of the treatment period ($P<0.005$) (Fig. 4A).

Fosinopril and losartan, at this low dosage, led to a significant decrease in the development of atherosclerotic plaques in the aortic arch of E mice.
content of MDA, respectively. Hydralazine had no significant effect on LDL oxidation (Fig. 6A).

Kinetic analysis of LDL oxidation with a low dose of fosinopril or losartan demonstrated a similar antioxidative effect as a high dose of fosinopril or losartan. After 10 weeks of treatment, the lag time for the initiation of CuSO$_4$-induced LDL oxidation was observed after 100 min for LDL derived from the placebo-treated group; whereas in the low dose fosinopril- or losartan-treated groups, no oxidation was observed up to 150 min ($P < 0.001$) (Fig. 6B). Hydralazine had no significant effect on the lag time required for LDL oxidation, compared with the placebo group (Fig. 6B).

4. Discussion

The present study demonstrates that the ACE inhibitor, fosinopril, significantly attenuates the development of atherosclerotic lesions in apo E deficient mice. This effect was associated with a significant reduction in plasma ACE activity, the propensity of their plasma and lesional LDL to oxidative modification and blood pressure.

Previous studies that assessed the potential preventive effects of ACE inhibitors were carried out in several animal models of atherosclerosis [8–11]. In hyperlipidemic hamsters, receiving a high cholesterol diet, Aberg et al. demonstrated that fosinopril significantly reduced the number of intimal macrophage-foam cells per mm and fatty streak area 85% and 90%, respectively. The antiatherogenic effect of fosinopril was related to a direct inhibition of ACE activity and Ang II formation, and also to some lowering of plasma cholesterol, a phenomenon that was not demonstrated in the present as well as in other studies [10,11,44,45].

Fosinopril significantly reduced plasma ACE activity in E$_0$ mice and this effect can be directly responsible for the attenuation of atherosclerosis by inhibiting Ang II-dependent effects such as vasoconstriction, macrophage foam cell formation, smooth muscle cell proliferation, enhanced monocyte adhesion to endothelial cells, inhibition of macrophage migration and increased oxidative stress [3–5,15,46]. In the aortic arch of hamsters, the presence of ACE mRNA and protein in endothelial cells, intimal macrophages and medial smooth muscle cells suggests that Ang II production occurs within the atheroma and this vasoactive peptide may directly promote the progression of the disease [12]. Therefore, reducing Ang II production with ACE inhibitors may decrease monocyte recruitment to the arterial wall and reduce foam cell formation.

In parallel to the attenuation of atherosclerosis, fosinopril significantly reduced the susceptibility of the mouse plasma LDL to oxidative modification, as shown by a significant reduction in the LDL-associated TBARS, as well as by prolongation of the lag time required for the

3.2.3. LDL oxidation

The susceptibility of LDL obtained from the apo E deficient mice to undergo lipid peroxidation was determined in mice treated with a low dose of fosinopril, a low dose of losartan or with hydralazine. After 10 weeks of treatment, the susceptibility of the mice LDL to lipid oxidation following its incubation with CuSO$_4$ was significantly reduced in the apo E deficient mice that were treated with low dose fosinopril or low dose losartan, in comparison with the placebo group, as shown by a 90% and 50% reduction ($P < 0.001$ and $P < 0.005$) in the LDL

lesions. The average lesion area was significantly lower in the low dose fosinopril-treated group (15 300±2167 μm$^2$), in the low dose losartan-treated group (18 300±2912 μm$^2$), in comparison with the placebo-treated group (25 200±1899 μm$^2$) ($P < 0.01$). On the other hand, in the hydralazine-treated group the lesion area was increased not significantly to 37 100±4714 μm$^2$, in spite of significant blood pressure reduction by this drug (Figs. 4B, 5).
initiation of LDL oxidation. Since studies in humans and animal models have shown that the oxidative modification of LDL plays a crucial role in the build-up of the atherosclerotic lesions, leading to coronary artery disease [17–23], the inhibition of LDL oxidation by fosinopril may contribute to the attenuation of atherosclerosis. Furthermore, since LDL oxidation takes place mainly in the arterial wall [47], the inhibitory effect of fosinopril on the oxidative state of LDL-like particles that were isolated from the mouse aortic lesions further suggests a direct effect of the drug on arterial wall Ang II production and LDL oxidation. We have shown previously that other antioxidants such as vitamin E may have an antiatherogenic effect in E0 mice [48]. On the other hand, in humans the efficacy of vitamin E as antiatherogenic was demonstrated only in the ‘CHAOS’ trial [49] by using a high-dose of vitamin E (400 IU daily). Other antioxidants such as β-carotene were not proven to be useful in prevention of atherosclerosis.

A high dose of fosinopril also significantly reduced the blood pressure in apo E deficient mice. This effect may also contribute to the inhibition of atherosclerosis.

Hypertension is recognized as a potent promoter of atherosclerosis in humans [50] as well as in hypercholesterolemic animals [51]. Epidemiologic data have shown that human subjects with low blood pressure also have low rates of clinical complication of atherosclerosis [52]. Many of the arterial changes induced by hypertension mimic those caused by hypercholesterolemia, including impaired endothelium-dependent relaxation, increased smooth muscle cell proliferation and connective tissue deposition [53–55]. Despite these known effects of elevated blood pressure on the arterial wall, little is known regarding the influence of the reduction of blood pressure on arterial responses to hypercholesterolemia.

In order to determine whether the antiatherosclerotic effects of ACE inhibitors could be dissociated from their blood pressure lowering effect, apo E deficient mice were treated with a low dose of fosinopril that was still adequate to reduce plasma ACE activity and LDL propensity to lipid peroxidation but was insufficient to lower blood pressure. We found that low dose of fosinopril also inhibited the progression of atherosclerosis in apo E deficient mice, although to a lesser extent than a high dose of fosinopril (where significant blood pressure lowering was observed). However, a significant reduction of blood pressure by hydralazine was not accompanied by an attenuation of atherosclerosis in our model. Similar to our present findings, recent studies in cholesterol-fed rabbits given enalapril [27] and hamsters treated with captopril [12] have suggested that the protection against atherosclerosis by ACE inhibitors in these models may be independent of blood pressure lowering.

In contrast to the present findings, Chobanian et al. [56] demonstrated that a low dose of the ACE inhibitor, trandolapril, which has no influence on blood pressure, had no effect on atherosclerosis in WHHL rabbits. The reason for the apparent difference in the results is unclear and...
Taken together, our data suggest that the antiatherosclerotic effects of fosinopril (and of losartan) in apo E deficient mice are due not only to blood pressure reduction but also to a direct inhibition of Ang II actions in the arterial wall, including inhibition of LDL lipid peroxidation.

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