Influences of α-tocopherol on cholesterol metabolism and fatty streak development in apolipoprotein E-deficient mice fed an atherogenic diet

Abstract

Although the role of oxidized lipoproteins is well known in atherogenesis, the role of vitamin E supplementation is still controversial. There is also little information about cholesterol metabolism (hepatic concentration and fecal excretion) in the new models of atherosclerosis. In the present study, we evaluated the effect of moderate vitamin E supplementation on cholesterol metabolism and atherogenesis in apolipoprotein E (apo E)-deficient mice. Apo E-deficient mice were fed an atherogenic diet containing 40 or 400 mg/kg of α-tocopherol acetate for 6 weeks. Total cholesterol in serum and liver and 3-OH-α-sterols in feces, and fecal excretion of bile acids were determined and histological analyses of aortic lesion were performed. A vitamin E-rich diet did not affect body weight, food intake or serum cholesterol. Serum and hepatic concentrations of cholesterol as well as sterol concentration in feces were similar in both groups. However, when compared to controls, the α-tocopherol-treated mice showed a reduction of about 60% in the atherosclerotic lesions when both the sum of lesion areas and the average of the largest lesion area were considered. These results demonstrate that supplementation of moderate doses of α-tocopherol was able to slow atherogenesis in apo E-deficient mice and to reduce atherogenic lipoproteins without modifying the hepatic pool or fecal excretion of cholesterol and bile acids.

Key words
- Antioxidants
- Atherosclerosis
- Apo E
- Mice
- α-Tocopherol
- Cholesterol

Introduction

Atherosclerosis is one of the major causes of morbidity, disability and premature mortality in industrialized countries. The oxidative hypothesis postulates that the presence and accumulation of oxidized low-density lipoproteins (LDL) in the subendothelium start a cascade of a complex sequence of events that can culminate in atheroma development (1,2). Human consumption of food rich in antioxidants is associated with a lower risk of atherosclerosis (3,4). Dietary antioxidants such as vitamin C, vitamin E, β-caro-
tene, flavonoids, isoflavonoids and organo-
sulfur compounds protect LDL against in
vitro oxidation (5).

Vitamin E is a non-enzymatic antioxidant present in plasma and cells that neutralizes free radicals. In ex-vivo experiments, supplementation with α-tocopherol significantly reduced the copper-mediated oxidation of human LDL (6). However, the reduction of atherosclerotic lesions by vitamin E in experimental models is still controversial (7-10). There is little knowledge of the mechanisms involved in this protection as well as of the levels of vitamin E necessary. The doses of vitamin E administered (800-2000 IU/kg of diet or 40 to 100 times higher than the recommendation for mice) in most of the experimental studies are higher than those used for human supplementation (400-800 IU/day). Few studies have tested the effects of vitamin E supplementation at lower concentrations. The amount supplemented in our study was about 20 times higher than the recommendation for mice and equivalent to a human intake of 200 IU/day. We believe that the results obtained with this level of supplementation will be closer to those obtained with the usual human supplementation.

The use of apolipoprotein E (apo E)-deficient mice in this study is justified because they develop lesions similar to those seen in humans and this animal model has been well accepted as an important tool to observe the effects of vitamin E not only on cholesterolemia but also on the evolution of plaques in different stages of the lesion.

The aim of the present study was to evaluate the rate of development of atherosclerotic lesions in apo E-deficient mice supplemented with moderate doses of vitamin E for short periods of time.

Material and Methods

Animals and experimental design

The homozygous apo E-deficient (apoE-/-) mice (of C57BL/6 background) (11,12) were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and kept in the animal facility of Instituto de Ciências Biológicas, UFMG, Belo Horizonte, MG, Brazil. Forty 7-week-old apoE-/- mice were used in this experiment. ApoE-/- mice were fed a control diet (N = 20) or an α-tocopherol-rich diet (N = 20) for 6 weeks. The animals were distributed based on their initial weight (16.5 ± 1 g) and initial serum cholesterol (470 ± 60 mg/dl). The mice were housed in groups of 4-6 in plastic cages and kept in a room with a controlled 12:12-h light/dark cycle. Free access to food and water was provided. The basic composition (g/100 g) of the atherogenic diet was: sucrose (50.2), casein (20), lard (15), salt mixture (5), cellulose (5), vitamin mixture (1), soybean oil (1), choline (1), cholesterol (1), cholic acid (0.5), and methionine (0.3). α-Tocopherol acetate (20 and 400 mg/kg) was added to the control and tocopherol-rich diets, respectively. The diets were prepared every two weeks and stored in the dark at -4ºC. Individual body weights were recorded weekly. At the end of the experiment, the animals were fasted for 8 h and killed under ether anesthesia. Blood was collected from the orbital plexus and serum was stored in a nitrogen atmosphere. Serum was frozen at -70ºC for further tocopherol determination. The aorta was removed from the root in the heart to the iliac bifurcation and fixed for histopathological analysis. The liver was removed, weighed and frozen at -20ºC. Feces collected during the last seven days of the experimental period were pooled, dried, weighed and powdered.

Analytical methods

Blood samples were centrifuged at 12,000 g (3,500 rpm) for 10 min and the sera were used for cholesterol determination on the same day. For tocopherol determination, sera were kept in a N2 atmosphere and stored at -7ºC. Total vitamin E, tocopherols and
tocotrienols in the diets and α-tocopherol in the serum were determined by high-performance liquid chromatography (13).

Total cholesterol in serum (14) and liver and 3-OH-α-sterols in feces were determined enzymatically using commercial kits (Katal, Belo Horizonte, MG, Brazil). Sera from apoE−/− mice were diluted 1:10 in 0.85% NaCl before cholesterol determination to keep the absorbance within the proper range of the test.

Fecal bile acids were extracted by the method of van der Meer et al. (15) and the supernatants were enzymatically assayed as previously described (16). Hepatic and fecal total lipids were extracted by the method of Folch et al. (17), gravimetrically quantified, resuspended in isopropanol and assayed for total cholesterol.

The heart and proximal section of the aorta were removed from the animals and cleaned of adventitial tissue. The top half of the hearts was obtained under stereoscopic observation and fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate-buffered saline at room temperature. The specimens were routinely processed for paraffin embedding and histological sections were examined under a light microscope by one individual who did not have access to the codes. The entire specimen was analyzed (250 sections per mouse). The aortic root area was recognized by the proximal presence of aortic valve leaflets. Every consecutive section (5 µm thick) throughout the aortic root area (300 µm - 60 sections per mouse) was taken for analysis and stained with hematoxylin and eosin. Of every five sections, one was kept for morphometric analysis using an image analyzer (KS 300 program) attached to a microcamera and Zeiss microscope.

The lesion area of each animal represents the sum of the lesion area obtained from the ten sections analyzed.

In a second experiment, fourteen 4-µm aortic sections (from aortic root) separated by 200-µm intervals were obtained and stained with hematoxylin-eosin. Morphological and morphometric analysis was performed using the average of the three largest lesion areas from each animal.

**Statistical analysis**

The Student t-test was used to compare independent values. P<0.05 was accepted as statistically significant.

**Results**

There were no differences in final body weight or total food intake between groups (data not shown). The concentration of α-tocopherol was nearly ten times higher in the experimental diet than that in the control diet (Table 1). As expected, α-tocopherol was the major component of total vitamin E in both diets. α-Tocopherol levels were two-fold higher in serum of animals receiving the α-tocopherol-enriched diet than in controls (Table 1).

At the end of the experiment, apoE−/− mice fed the control and α-tocopherol diets had similar serum cholesterol levels (Table 2). No significant differences were found in total lipid, triacylglycerol (data not shown) or cholesterol concentrations in liver (Table 2). Histopathological analysis showed similar fat accumulation in livers of both groups (data not shown).

Hepatic cholesterol and fecal bile acid and 3-OH-α-sterols were determined to investigate if the reduction of plasma choles-

| Table 1. Dietary tocopherols and serum α-tocopherol of apoE−/− mice fed an atherogenic diet containing 40 IU/kg (control) or 400 IU/kg α-tocopherol acetate for 6 weeks. |
|---------------------------------|-----------------|-----------------|
| α-Tocopherol (mg/kg of diet)     | 38.1 ± 3.6       | 360.2 ± 18.8    |
| δ-Tocopherol (mg/kg of diet)     | 0.5 ± 0.0        | 0.5 ± 0.0       |
| γ-Tocopherol (mg/kg of diet)     | 6.6 ± 0.3        | 21.0 ± 0.7      |
| Vitamin E (IU/kg of diet)        | <0.1             | <0.1            |
| Serum α-tocopherol (mg/dl)       | 40               | 400             |

terol in the α-tocopherol group was due to an alteration in cholesterol catabolism. Since sitosterols are not important components of our atherogenic diet, the concentrations of 3-OH-α-sterols reflect concentrations of cholesterol and its catabolic products in feces. No differences were observed in hepatic or fecal parameters (Table 2).

Atherosclerotic lesions were found in both groups. However, the control group had a five-fold increase in the average sum of lesion areas when compared with the α-tocopherol group (546,115 ± 338,545 and 110,203 ± 49,293 µm², respectively) (Figure 1).

When the average area of the largest lesion of each animal was considered, the lesion area was four-fold higher in the control group compared with the α-tocopherol group (Figure 2).

Control animals had intermediate to advanced lesions with the presence of several components such as necrotic core, cholesterol clefts and fibrous cap (Figure 2A and C). α-Tocopherol supplementation was able to reduce the lesion area as well as calcium and cholesterol deposits and necrotic core (Figure 2B and D). The predominant lesions observed in this group were fatty streaks and intermediate lesions with foam cells deposited in intima and media. Therefore, α-tocopherol supplementation resulted in a reduction in both size and development of lesions: the necrotic core was reduced, and the calcium and cholesterol cleft depositions were impaired compared to control.

Table 2. Serum and hepatic cholesterol, fecal 3-OH-α-sterols and bile acids of apoE-/- mice fed atherogenic diets containing 40 IU/kg (control) or 400 IU/kg α-tocopherol acetate for 6 weeks.

<table>
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<tr>
<th></th>
<th>Control group</th>
<th>α-Tocopherol group</th>
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<tbody>
<tr>
<td>Serum cholesterol (mg/100 ml)*</td>
<td>2593 ± 172</td>
<td>2274 ± 249</td>
</tr>
<tr>
<td>Hepatic cholesterol (mg/g liver)*</td>
<td>24.12 ± 2.65</td>
<td>18.74 ± 1.58</td>
</tr>
<tr>
<td>Fecal bile acids (µmol/g feces)+</td>
<td>23.15 ± 7.90</td>
<td>11.50 ± 1.25</td>
</tr>
<tr>
<td>Fecal 3-OH-α-sterols (mg/g feces)+</td>
<td>40.35 ± 3.80</td>
<td>47.50 ± 1.75</td>
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</tbody>
</table>

*Mean ± SEM of individual values (N = 18).
+Mean ± SEM of 3 pooled samples.

Discussion

The higher levels of α-tocopherol in plasma of animals from the α-tocopherol group (Table 1) confirm the efficiency of dietary supplementation with α-tocopherol for raising the plasma levels of vitamin E. However, the increase was not proportional to the dietary content of the vitamin. Yoshida et al. (18) isolated and characterized a tocopherol-binding protein from cytosol of rat liver. This protein may incorporate α-tocopherol in very-low-density lipoprotein (VLDL) particles (19,20). The other forms of vitamin E are similarly absorbed and reach the liver in chylomicron remnants, being excreted into the bile with excess of α-tocopherol (20,21). There is a quantitative limitation in tocopherol-binding protein incorporation of α-tocopherol in VLDL that could be explained by the inability to raise plasma
levels of α-tocopherol beyond two to four times even after excessive ingestion (21). The levels found in the control group (1.22 mg/dl or 28.37 µM) are similar to those reported by Praticò et al. (22) for a group that was not supplemented with α-tocopherol (1.29 mg/dl or 30 µM), a fact that can be explained by the similar levels of vitamin E in the diet (40 IU/kg).

After 6 weeks of supplementation with α-tocopherol, the serum levels of cholesterol were similar to the control animals. Although reduction of cholesterol induced by vitamin E has been described in rabbits (9,23) and C57BL/6 mice (24), many authors have reported no cholesterol-lowering effect of dietary vitamin E (7,10,23,25,26). These reports differ from one another in many respects such as animal models, levels of dietary vitamin E, extent of supplementation, type of diets and simultaneous administration of other antioxidants - selenium, β-carotene, and vitamin C (27-29).

Conflicting data have been reported concerning the effect of vitamin E on cholesterolemia in humans. Hermann et al. (30) supplemented human subjects with 600 IU of α-tocopherol for 30 days; an increase in HDL-cholesterol and no change in triglycerides or total cholesterol occurred. These results were not confirmed by other investigators who used the same experimental protocol (31,32).

Data of cholesterol metabolism in apo E knockout mice are scarce. Since differences in the effect of vitamin E on serum cholesterol can be attributed to changes in cholesterol metabolism, we investigated cholesterol excretion and storage in liver. No differences were seen in hepatic cholesterol, its fecal catabolic products or fecal bile acids between groups.

In apo E knockout mice atherosclerosis is associated with an increase of atherogenic fractions of lipoproteins and lipid peroxidation in plasma as well as their susceptibility to peroxidation in conditions of oxidative stress (22,33-36). Knockout mice fed a commercial diet supplemented with 2000 IU of vitamin E/kg for 16 weeks had significantly lower levels of isoprostanes in urine and plasma when compared with non-supplemented animals (22). The supplementation also significantly reduced the area of lesion in the aorta of knockout mice. Thus, it seems that oxidative stress may be reduced by *per os* administration of vitamin E to these mice.

These results demonstrate that supplementation with vitamin E delays the development and formation of fatty streaks in apo E-deficient animals and reinforce the hypothesis that supplementation with α-tocopherol...
phol exerts beneficial effects in terms of prevention of atherosclerosis.

Acknowledgments

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