Effect of vitamin E supplementation on antibody levels against malondialdehyde modified LDL in hyperlipidemic hamsters

Fernando G. de Oliveira, Cláudio L. Rossi, Marcelo G. de Oliveira, Mário J.A. Saad, Lício A. Velloso

Abstract

Objective: The aim of this work was to investigate the effect of vitamin E (VE) supplementation on the formation of autoantibodies against oxidized low density lipoproteins (LDL) in a hyperlipidemic animal model. Methods: Thirty-four male hamsters (Mesocricetus auratus), 4 weeks old, were divided into three groups: Group A (n=9) was fed with standard rodent chow; group B (n=13) was fed with a standard rodent chow plus 2% cholesterol and 10% butter and group C (n=12) was fed with the same diet plus 0.2% (w/w) VE. Blood samples were collected by intracardiac puncture and antibody levels were determined in each animal at 4 weeks of age and after 20 weeks of experimental diet. A modified ELISA technique was used to analyze the modulation of autoantibody titers against an epitope of oxidized LDL in serum samples. Antigens prepared for the ELISA tests were characterized using spectrofluorimetry. Serum VE levels were determined in the lipidic fractions by HPLC. Results: The groups fed with cholesterol-fat enriched diet presented a three-fold increase in total serum cholesterol and two-fold increase in serum triglycerides compared to the control group. VE supplementation played no role in serum cholesterol and serum triglyceride concentrations but led to a decreased autoantibody (anti-LDL–malondialdehyde) formation (P<0.05). Conclusions: Our results show that VE supplementation leads to a lower production of autoantibodies against oxidized LDL, suggesting a protective effect of VE against in vivo oxidation of LDL particles, in a dose-dependent manner.

Keywords: Atherosclerosis; Lipoproteins; Cholesterol; Free radicals

1. Introduction

Much evidence suggests that low density lipoprotein (LDL) particles undergo oxidative modifications in vivo [1–5], leading to an increased atherogenicity by several mechanisms, such as enhanced uptake by macrophages [4], chemotactic activity for circulating monocytes [6], inhibition of macrophage motility [7], cytotoxicity for endothelial cells [8,9], and induction of endothelial dysfunction [10]. During oxidative modification of LDL, highly reactive lipid peroxidation products, such as malondialdehyde (MDA) form adducts with free amino groups of lysine of apolipoprotein B (apo B). Modified apo B is highly immunogenic, and circulating autoantibodies to epitopes of oxidized LDL, such as MDA–lysine, have been demonstrated in plasma and in atherosclerotic lesions of humans and animals [1,5]. The titer of these autoantibodies may reflect the extent of the LDL oxidation in vivo, serving as a marker of the process. The development of autoantibody titers correlates with the extent of atherogenesis in cholesterol-fed receptor-deficient mice [11]. A number of studies have suggested that higher titers of these autoantibodies are found in patients with increased progression of carotid atherosclerosis [12], peripheral vascular disease [13] and diabetes [14], and may be used to predict myocardial infarction [15]. Vitamin E (VE), particularly α-tocopherol, is a first line natural defense against LDL oxidation [16].

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and VE supplementation has been demonstrated to decrease aortic lesions and aortic levels of MDA in hyperlipidemic rabbits [17], as well as to decrease LDL oxidability in humans [18]. More recently, Stephens et al. [19] have shown that VE supplementation may significantly reduce the risk of myocardial infarction in a prospective controlled study of 2002 patients with angiographically proven coronary atherosclerosis.

In the present work, we have analyzed the effect of VE supplementation on the modulation of anti-MDA–LDL autoantibody formation in an animal model of hyperlipidemia. Our results indicate that VE supplementation may provide a partial protection to LDL particles from in vivo oxidation, mitigating the increase in the serum levels of anti-MDA–LDL.

2. Methods

2.1. Animals

Thirty-four male hamsters (Mesocricetus auratus), 4 weeks old, were used in the experiments. The animals were obtained from the medicine department (Botucatu, SP, Brazil) and housed under an artificial lighting cycle of 12-h light–dark periods. Food and water were provided ad libitum and individual ponderal curves were registered along the study. The animals were divided into three groups: Group A (n=9) was fed with standard rodent chow; group B (n=13) was fed with a standard rodent chow (Nuvilab CR1-Nuvital, Curitiba, Brazil) plus 2% cholesterol and 10% butter and group C (n=12) was fed with the same diet as group B plus 0.2% (w/w) VE as α-tocopherol acetate (Ephynol®, Roche, Jacarepagua, SP, Brazil). The antibody levels were determined in each animal at 4 weeks of age (while on standard rodent chow) and after 20 weeks of experimental diet. In both occasions, the animals were anesthetized with sodium thiopental (Thionembutal Abbott, São Paulo, Brazil); blood samples were collected by intracardiac puncture after 12 h fasting and serum was stored at −80°C. The experimental procedures were approved by the Local Animal Care Committee and conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No 85-23, revised 1996).

Originally, the number of animals assigned to each group was: group B, n=13, group C, n=13 and control group A, n=10. Two animals died in the first cardiac puncture, one in group C and one in group A.

2.2. Enzyme-linked immuno sorbent assay for antibodies against oxidized-LDL

Because MDA-modified-LDL (MDA–LDL) has been reported to represent a prominent epitope of oxidized-LDL (ox-LDL) [20], MDA–LDL was used as an antigen for detecting autoantibodies generated against ox-LDL. The MDA–LDL was prepared as described by Palinski et al. [21], with minor modifications. Fresh MDA was generated from malonaldehyde bis(dimethylacetal) (Aldrich, Milwaukee, WI, USA), by acid hydrolysis. Lyophilized human LDL (Sigma, St. Louis, MO, USA) was incubated with freshly prepared MDA (100 µl/mg of LDL) at 37°C for 3 h. The MDA–LDL was then extensively dialyzed against phosphate buffered saline (PBS), pH 7.4, containing 1 mmol/l EDTA. Protein was measured in the dialyzed preparation by the Biuret method (Bio-Rad, Hercules, CA, USA). The MDA–LDL was then dissolved in the coating buffer (carbonate/bicarbonate 50 mmol/l, pH 9.7) to a final protein concentration of 0.05 µg/ml, and 100 µl of this solution was added to each well of ELISA polystyrene plates (Corning Inc., Corning, NY, USA). The plates were incubated 90 min at 37°C and then overnight at 4°C. After washing twice with PBS, pH 7.4, containing 0.05% poly(oxyethylene sorbitan) monolaurate (TWEEN 20, Sigma) the plates were blocked with PBS, pH 7.4, containing 0.1% Tween 20 and 2% bovine serum albumin (BSA) Fraction V (Calbiochem—Novabiochem, La Jolla, CA, USA) at 37°C for 3 h. After removing the blocking solution, the plates were stored at −20°C until use. To perform the ELISA, the plates were defrosted, washed four times, and serum samples were added (100 µl/well, diluted 1:11 in PBS–0.1% Tween 20–0.05% BSA) and incubated at 37°C for 4 h. After washing three times, the plates were incubated with monoclonal antibody anti-hamster IgG and IgM (Sigma) diluted 1:500 in PBS–0.1% Tween 20–0.05% BSA, at 37°C for 90 min. The plates were then washed three times, and peroxidase-conjugated rabbit anti-mouse IgG (whole molecule) antibody (Sigma), diluted 1:500 in PBS–0.1% Tween 20–0.05% BSA was added, and the mixture was incubated for 90 min at 37°C. After washing four times, the plates were developed using the substrate 3,3’,5,5’-tetramethylbenzidine dihydrochloride (TMB, Sigma) diluted in acetate–acetic acid buffer, 0.1 mol/l, pH 6.1, plus hydrogen peroxide 0.01% (100 µl/well), incubated for 30 min at 37°C in the dark, followed by the addition of sulfuric acid 1 mol/l, 100 µl/well. The absorbance was read at 450 nm in an automatic ELISA microplate reader (Multiskan MS Type 352, Labsystems, Finland). Each sample was tested in triplicate, and the results were expressed as mean optical density values subtracted from the mean blank value (arbitrary units). The intra-assay coefficient of variation was <4%.

2.3. Determination of serum levels of vitamin E

The lipidic fraction was extracted from the samples according to Stewart-Lee et al. [22]. A high-performance liquid chromatographic method was developed using a Waters 600-E liquid chromatograph with a U6K injection valve, a 600 E pump, a model 484 variable wavelength
UV–vis detector and a 4400 integrator (Waters, chromatography division, Milford, MA, USA). A reversed-phase column μ-Bondapack C$_{18}$ (3.9×150 mm) (Waters) was used. The mobile phase consisted of pure acetonitrile for HPLC (Carlo Erba, Rodano, Italy). Triplicate 10-μl injections were made of each analyte. Detection was carried out at 286 nm and peak maxima were taken as the retention times. Results were plotted on a calibration curve obtained with α-tocopherol acetate standards in the range 2–75 μg/ml. Peak areas were taken to calculate the VE concentrations.

Lipid-standardized serum levels of vitamin E were calculated as the rate of vitamin E/total lipids (TLs), (μgVE/mgTL) where TLs correspond to the total serum male hamsters divided into three groups, at the end of the experimental period. Hamsters were fed with control diet containing 2% cholesterol and 10% butter (group B), the same diet as group B plus 0.2% vitamin E (group C), and control diet (group A); $P(A \times B \times C) < 0.0001$ for total serum cholesterol and $P(A \times B \times C) = 0.01$ for triglycerides.

2.4. Other analytical procedures

The antigen prepared for the ELISA tests (MDA–modified LDL) was characterized by spectrofluorimetric analysis using a spectrofluorimeter Aminco SPF-500C (SLM Instruments ICC, Urbana, IL, USA) with excitation at 400 nm and emission read at 465 nm [16].

Total serum cholesterol and triacylglycerols were measured using an enzyme-based method (Labtest Diagnóstica, Lagoa Santa, Brazil).

2.5. Statistical analysis

All data are expressed as mean±SEM. Statistical significance was evaluated using the one-way ANOVA test for comparisons among three groups ($A \times B \times C$) and the Student’s $t$-test in the cases where the comparison between two means was appropriate. A value of $P \leq 0.05$ was considered statistically significant. The statistical analysis were carried out by using the statistical software spss 7.5 for Windows and Microsoft ORIGIN, version 3.5.

3. Results

There were no statistical differences in body weight between the three groups at the beginning, and at the end of the experimental period (Table 1).

<table>
<thead>
<tr>
<th></th>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
</tr>
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<tbody>
<tr>
<td>Initial weight (g)</td>
<td>161±4.1</td>
<td>154±6.3</td>
<td>161±5.3</td>
</tr>
<tr>
<td>Final weight (g)</td>
<td>178±4.3</td>
<td>164±5.7</td>
<td>176±4.8</td>
</tr>
</tbody>
</table>

Total serum cholesterol at the end of the experimental period was significantly higher in group B ($P=0.003$) and C ($P=0.0002$) than in group A; serum triglycerides was significantly higher in group B ($P=0.00002$) and C ($P=0.003$) than in group A. There were no significant differences in serum lipids between groups B and C (Fig. 1).

Serum levels of VE (Fig. 2) were greater in group C as compared to groups B ($P=0.0001$) and A ($P=0.002$), with no differences between groups A and B; $P(A \times B \times C) = 0.00001$.

Fig. 2 also shows the lipid-standardized serum levels of VE (LSSL-VE), which provide an estimate of the VE content of lipoproteins, at the end of the experimental period.
period. It can be seen that the LSSL–VE was higher in Group C than in groups B \((P=0.00006)\) and A \((P=0.03)\). In addition, the LSSL–VE was also significantly higher in group A than in group B \((P=0.009)\); \(P\) (A×B×C)=0.00006.

No significant differences in serum anti-LDL–MDA antibody levels were observed between the three groups at the beginning of the experimental period. However, at the end of the experimental period, these levels were higher in group B and C \((P<0.001)\) than in group A, while there was no significant differences between groups B and C; \(P\) (A×B×C)=0.003.

All groups showed a significant increase in the levels of autoantibodies. In addition, the absolute difference in the levels of autoantibodies from the beginning to the end of the experimental period was statistically different between groups B and C \((P<0.05)\), and between groups B and A \((P=0.006)\), but not between groups C and A; \(P\) (A×B×C)=0.01 (Fig. 3).

In the groups not supplemented with VE (A and B), there was a significant correlation between the final levels of anti-LDL–MDA antibodies and total serum cholesterol \((P=0.01, r=0.54)\) (Fig. 4A), serum triglycerides \((P=0.015, r=0.52)\) (Fig. 4B) and total lipids \((P=0.004, r=0.6)\) (Fig. 4C). Finally, the analysis of all groups together showed a significant inverse correlation between the absolute variation in the antibody levels (final−baseline titles of anti MDA–LDL antibody) and lipid-standardized log of VE \((\text{log } VE\text{ divided by total lipids})\), with \(P=0.007\) and \(r=-0.46\) (Fig. 4D).

The fluorimetric analysis showed that MDA–LDL have an emission band with maximum at 465 nm whilst LDL, MDA and PBS solutions alone, do not present any emission band in the range 400–600 nm (data not shown).

These spectra confirm that the emission at 465 nm is characteristic of the adduct MDA–LDL.

4. Discussion

The results obtained with the animal model used in this work, have shown that a cholesterol–fat enriched diet, led to a substantial increase in serum lipids, and to an increase in the autoantibody formation against MDA–LDL. Our data are consistent with the results obtained by Palinsk et al. \([11]\) who found similar increases in autoantibody formation, in rabbits treated with cholesterol–fat enriched diets. Recently, Hulthe et al. \([23]\) reported data showing no significant differences in antibody titers against ox-LDL or MDA–LDL, between a group of patients with familial hypercholesterolemia and a control group. However, in such work all but three hypercholesterolemic patients underwent cholesterol-lowering therapy, 65% of them with pravastatin, which has recognized antioxidant properties, attenuating oxidative susceptibility of LDL in hypercholesterolemic patients, which makes interpretations difficult \([24]\).

We have seen that the addition of VE to a cholesterol–fat enriched diet did not modify the increase in serum levels of cholesterol and triglycerides. However, VE reduced the formation of specific anti-MDA–LDL antibodies. It is probable that the greater the serum levels of lipoproteins, the more intense will be the oxidative modifications of LDL particles. It is known that LDL particles cross the endothelium to the intimal space in direct proportion of their serum concentration. Therefore, it is more likely that oxidative modifications of LDL occur primarily in the intima microdomains, protected from the various antioxidants found in plasma \([10]\). Despite the similar levels of VE, found between groups A and B, the lipid standardized serum levels of VE were significant lower in group B than in group A in the present model. This result shows that, the greater is the serum level of lipoprotein, the lower is the relative content of VE per particle of LDL, and consequently the lower will be its antioxidative activity upon specific substrates.

The humoral immune response (as measured by the titer of plasma autoantibodies) is regulated by a diversity of mechanisms, and does not reflect only antigen burden. The relation between serum total lipids and serum anti MDA–LDL levels observed in the present work provides indirect evidence of in vivo oxidation of LDL. The significantly lower increase in the autoantibody levels of the supplemented animals (Fig. 3) and the inverse logarithmic correlation between lipid-standardized VE and antibody anti-MDA–LDL increase (Fig. 4), observed in this work, point to a protective effect of VE supplementation in the reduction of the oxidative modification of LDL in hyperlipidemic conditions.

In addition to its action as an inhibitor of LDL oxida-
tion, VE may modulate the immune response. In a recent randomized controlled trial, VE supplementation enhanced cell-mediated and antibody response following hepatitis B, tetanus and diphtheria vaccination in healthy elderly subjects [25]. Thus, the results observed in the present study may represent a combination of the effects of VE upon antigen formation and immune system activation, resulting in an apparent milder autoimmune response. The role of autoantibodies in the atherogenic process, however, remains elusive. Some authors [26,27] have described accentuation of in vitro formation of foam cells when antibodies against modified LDL or apolipoprotein B100 were added to modified LDL or LDL aggregates, suggesting an atherogenic role to these autoantibodies. On the other hand, more important evidence came from two studies using immunization of animal models of hyperlipidemia with oxidized or MDA modified LDL, that showed a significant reduction in the extent of atherosclerotic lesions in aorta, and suggested a protective effect of these autoantibodies against the atherosclerotic process [28,29].

Apart from the role of the autoantibodies in the physiopathologic process, their measurement may also be useful as an indicator of the extent of LDL oxidation in vivo, or even of the atherogenic process itself. The modified ELISA technique described in the present study is faster and more straightforward than the usual ELISA technique described for this purpose, due to short incubation times at 37°C and the possibility of preparing several ELISA plates simultaneously, which can be kept frozen until use. With slight variations, most published assays of antibodies against different types of modified LDL are based on determining the difference or the ratio between the binding of a given sample to modified LDL and to native LDL.

However, as discussed by Virella et al. [30], this approach ignores the fact that the modification of LDL adds negative charges to the LDL molecule, increasing its potential for charge-dependent non-covalent interactions with IgG. This fact becomes relevant when considering that the antioxidized LDL autoantibodies isolated from human subjects appear to be predominantly of moderate-to-low affinity, and of variable cross-reactivity [31]. Moreover, Hulthe et al. [23] have demonstrated that ELISA plates coated with ‘native’ LDL probably exposes epitopes common with modified LDL, suggesting that during coating process (despite the presence of antioxidants) LDL is modified in

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\text{Total serum lipids (serum cholesterol + triglycerides) and total serum cholesterol and triacylglycerols were measured using an enzyme based method and expressed in mg/dl. Serum levels of anti-MDA–LDL were determined by ELISA, and expressed in arbitrary units.}
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\text{Vitamin E serum levels were determined by HPLC, and expressed in µg/ml. Total serum lipids (serum cholesterol + triglycerides) and total serum cholesterol and triacylglycerols were measured using an enzyme based method and expressed in mg/dl. Serum levels of anti-MDA–LDL were determined by ELISA, and expressed in arbitrary units.}
\]
some extent. For this reason, in our experiment, we performed an ELISA technique using the absolute quantification of the antibody titers, without other reference value but the mean blank value of each ELISA plate. In the present study, all the ELISA analysis were performed simultaneously, eliminating the need for a reference standard. However, to get acceptable reproducibility on this technique, in order to use it for clinical or experimental purposes, it will becomes necessary to use a standard purified anti-LDL–MDA antibody in progressive dilutions in each ELISA plates.

Finally, its must be stated that the average administration dosage used in group C was 131.25 I.U./kg/day or 87.5 mg/kg/day. Although this dosage can be considered high, if compared to the average clinical condition in humans, some works report clinical trials conducted in humans using VE dosages as high as 2000 I.U./day [32,33]. Other works have demonstrated an effective action of VE in reducing the risk of coronary disease only with the supplementation of more than 100 I.U./day [34]. The average level of serum VE achieved in our study, in group C animals (45.6 μg/ml, or 96.5 μmol/l) cannot be considered extremely high, if we take into account that unsupplemented humans were reported to present serum VE levels between 3.4 and 35.6 μg/ml [35], or as high as 76.4 μmol/l [36]. Such considerations may explain the lack of correlation between ingestion of VE (or VE serum levels), and coronary disease in humans unsupplemented or supplemented with low doses of VE, observed in other works. In this regard, the indication of a significant protective action of VE against the in vivo oxidation of LDL, obtained in the present work, can be viewed as an evidence in favor of the use of higher VE dosages in clinical trials.

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