Effects of Vitamin E and Vitamin C supplementation on plasma lipid peroxidation and on Oxidation of apo-lipoprotein B-Containing lipoproteins in experimental hyperthyroidism

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Abstract: Increasing numbers of experimental and epidemiological studies suggest the involvement of free radicals in the pathogenesis of various disease entities. Similarly, oxidative processes have been implicated as playing roles in the genesis of hyperthyroidism-induced damage. In this study, we investigated the effects of vitamin E and vitamin C on plasma lipid peroxidation and the susceptibility of apolipoprotein B (apo B)-containing lipoproteins to oxidation in experimental hyperthyroidism. The study animals were initially divided into a control group (Group C) and a hyperthyroid group. The latter was further re-grouped later according to their vitamin supplementation status: Hyperthyroid group without vitamin supplementation (Group H), hyperthyroid group with vitamin E supplementation (Group H+E) and hyperthyroid group with vitamin C supplementation (Group H+C).

Malondialdehyde (MDA) level was measured as an indicator of plasma lipid peroxidation. The apo B-containing lipoproteins were separated by precipitation and incubated with copper sulphate. The MDA levels of this non-HDL fraction were measured prior to and after 1, 2 and 3 hours of incubation. Plasma MDA levels showed no significant differences among groups. Whereas MDA levels measured in non-HDL fraction were significantly higher in Group H than Group C. Group H+E and Group H+C had significantly lower MDA levels than Group H in all these measurements. This finding strongly indicates an increased susceptibility of apo B-containing lipoproteins to oxidation in hyperthyroidism, and that vitamin E as well as vitamin C supplementation protect these lipoproteins from copper-induced oxidation. J. Med. Invest. 46 : 29-33, 1999

Key words : *hyperthyroidism, vitamin E, vitamin C, oxidized lipoproteins*

INTRODUCTION

Free radicals and other reactive oxygen species are continuously produced *in vivo*. The generation and consequence of free radicals in biological systems have received much attention recently. Lipids, carbohydrates, proteins and DNA are major target molecules for free radical attack (1, 2).

Aerobic organisms are protected against oxidative injury by an array of antioxidant defense systems. The radical scavenging antioxidants inhibit chain reaction initiation and/or break chain propagation. While water-soluble radical-scavenging antioxidants can inhibit chain initiation by removing aqueous radicals (3), lipophilic antioxidants such as vitamin E, ubiquinol and carotenoids can break chain propagation within lipophilic media (4, 5). Low-density lipoprotein (LDL) is an important target molecule for lipid peroxidation in plasma. Besides LDL, apolipoprotein B-containing lipoproteins [very low-density lipoprotein (VLDL), lipoprotein (a)] are also sensitive to oxidation (6).

There is some experimental and epidemiological

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evidence to suggest the involvement of free radicals in the pathogenesis of various diseases such as cancer, inflammation, aging and atherosclerosis (2). Increasing evidence shows that oxidative processes have a role in the genesis of hyperthyroidism-induced damage (7-10). One of the major effects of thyroid hormones is to increase mitochondrial respiration (11, 12), by altering the concentrations of the components in the electron-transport system, as well as by altering the redox state of the components. Accelerated mitochondrial electron transport, brought about by the thyroid hormone-induced hypermetabolic state, results in the increased generation of superoxide at the site of ubiquinones (13). Hyperthyroidism induces an increase in oxidative enzymes, mitochondrial superoxide dismutase (SOD) and in lipid peroxide levels, and a decrease in cytosolic SOD, glutathione peroxidase and catalase activities (14).

The present study was designed to evaluate the changes in apolipoprotein (apo) B-containing lipoprotein oxidation in hyperthyroidism as well as to investigate whether vitamin E and vitamin C can play a protective role in this case.

MATERIALS AND METHODS

Chemicals

A cholesterol enzymatic assay kit was obtained from Boehringer Mannheim (Germany) ; thiobarbituric acid from Sigma Chemical Co. (USA) ; ethylenediamine tetraacetic acid (EDTA-Na_2), copper sulphate ($CuSO_4$), NaCl, NaH_2PO_4, NaOH, $MgCl_2$, n-butanol and trichloroacetic acid (TCA) from Merck (Germany) ; dextran sulphate sodium salt (M.W 500.000) from Pharmacia Fine Chemicals (Sweden) ; 1,1,3,3-tetraethoxypropane from Fluca (Germany). Tri-iodothyronine (T_3) and thyroxine (T_4) radioimmunoassay kits were purchased from Amersham Int (UK).

Treatment of animals

In the study, male Sprague-Dawley rats weighing 350 to 450 g were used. They had free access to both standard rat chow and tap water during the entire study period of 36 days. While control animals (Group C, n=8) received only standard rat chow and tap water until the completion of the study, the remaining rats (n=23) were brought into hyperthyroid state by L-thyroxine administration at the dose of 40 μg/100 g body weight/day mixed into the water. On the 20 th day, hyperthyroid rats were subdivided into three groups. The first group (n=7) continued to receive only L-thyroxine (Group H), while second group (n=8) received vitamin E (Ephynal, Roche) supplementation at the dose of 150 mg/kg/day mixed into the chow (Group H+E), and the third group (n=8) received vitamin C (Redoxan, Roche) supplementation at the dose of 100 mg/kg/day in the water (Group H+C) along with the previously initiated L-thyroxine treatment. On day 36, blood samples were drawn into tubes containing EDTA. Na_2 (final EDTA concentration 4.08 mmol) after 8-10 hours of fasting by cardiac puncture under light ether anesthesia. Plasma was isolated by centrifugation at 1500 xg for 15 minutes at room temperature.

Isolation of non-HDL fraction

A combined procedure of isolation of the non-HDL fraction and the removal of EDTA was described by Phelps and Harris (15) and developed by Zhang et al (16). The non-HDL fraction was precipitated from twice diluted plasma by adding the precipitation reagents [Dextran sulphate (20 g/L) : magnesium chloride (2 mol/L) 1 : 1] and centrifuging at 1500 xg for 10 minutes. The non-HDL pellet was suspended in 0.9% phosphate-buffered saline (PBS) (0.15M NaCl, 10 mM NaH_2PO_4, pH : 7) and reprecipitated by adding precipitation reagent and centrifuging in order to remove EDTA. The reprecipitated non-HDL pellet was dissolved in 4% PBS (0.68 M NaCl, 10 mM NaH_2PO_4, pH : 7). Just after isolation of non-HDL and removal of EDTA, the EDTA-free non-HDL fraction was diluted with 4% PBS to a final cholesterol concentration of 200μg/mL. Adding copper sulphate at a final concentration of 0.046 mM started the oxidation. All the oxidation experiments were performed at 37°C At 60, 120 and 180 minutes, the lipid peroxide content of the non-HDL fraction was measured by thiobarbituric acid-reacting substances (TBARS) assay as described by Buege and Aust (17). TBARS were expressed as MDA equivalents content using 1,1,3,3-tetraethoxypropane as a standard (nmol MDA equivalents/mg non-HDL cholesterol).

Other measurements

Serum total T_4 and T_3 concentrations were measured using a commercial radioimmunoassay. Plasma malondialdehyde level was measured by the reaction of lipid peroxides with thiobarbituric acid (18).

Statistical Analyses

The SPSS/PC computer program was used for

statistical analysis. Results were expressed as mean ±SD. Statistical comparisons between the groups were performed by Kruskal-Wallis nonparametric ANOVA test. For all analyses, $p<0.05$ was regarded as significant.

RESULTS

The effect of hyperthyroidism on body weights is shown in Table I. The difference of initial weights of the rats was not statistically significant. However, at the end of the experiment, the weights of the hyperthyroid groups (H, H+E and H+C) were significantly lower compared with the control group. Group H+C rats weighed even less than any other hyperthyroid group.

Table Ⅰ. Body weights of the rat groups

Body weight, g	Control (n : 8)	Group H (n : 7)	Group H+E (n : 8)	Group H+C (n : 8)
Initial	414±19	422±18	408±34	400±20
Final	422±16	354±13[a]	358±29[a]	323±25[a]

[a] : p<0.001 compared with control rats.

Hyperthyroid induced rats (H, H+E, and H+C groups) had serum thyroxine levels of 11±1.9, 8.3 ±1.3 and 16.7±4.5 μg/dL respectively vs. 2.0± 1.0 μg/dL in control rats ($p<0.001$). Likewise, serum T_3 levels of Group H, H+E and H+C were significantly higher (165.7±18.4, 105.6±13.7 and 152.6 ±18.9 ng/dL, respectively) than that of Group C (57.1±33.0 ng/dL) (For p values, refer to Table II). Serum T_3 and T_4 levels were significantly lower in Group H+E animals compared with those in Group H. The Group H+C serum T_4 level, when compared with Group H, was found to be significantly elevated, although there was no difference between these two groups in respect to serum T_3 levels (Table II).

Table Ⅱ. Serum T_4 and T_3 levels in four rat groups

Analytes	Control	Group H	Group H+E	Group H+C
T_4, μg/dL	2.0±1.0	11.0±1.9[a]	8.3±1.3[a,d]	16.7±4.5[a,d,e]
T_3, ng/dL	57.1±33.0	165.7±18.4[a]	105.6±13.7[b,c]	152.6±18.9[a,f]

[a, b] : [a]p<0.001, [b]p<0.05 compared with control group
[c, d] : [c]p<0.001, [d]p<0.01 compared with Group H
[e, f] : [e]p<0.001, [f]p<0.05 compared with Group H+E (ANOVA)

Plasma MDA levels were slightly higher in all hyperthyroid animals (Groups H, H+E and H+C) compared with control rats, but the differences were not statistically significant (Table III).

Table Ⅲ. Plasma MDA levels in rats groups

Analyte	Control	Group H	Group H+E	Group H+C
MDA, nmol/mL	8.9±1.5	10.0±1.7	9.4±1.5	9.7±0.8

In all study groups, the non-HDL fractions incubated with $CuSO_4$ resulted in an increase in lipid peroxide content (increased TBARS amount as MDA equivalents) as shown in Table IV. The MDA levels of those non-HDL fractions were measured prior to (MDA baseline) and after 1, 2 and 3 hours of incubation. In the vitamin-supplemented groups (Groups H+E and H+C), the baseline MDA values were lower than in Group C and Group H. Only in Group H+E did the MDA level reach statistical significance ($p<0.01$). After the incubation for 1, 2 or 3 hours, the MDA levels were increased in both Group C and Group H, but the increments were greater in the latter. At the same time points, the MDA levels in Group H+E and Group H+C were significantly reduced compared to Group H animals ($p<0.01$), and the reduction was even more pronounced in Group H+E than Group H+C ($p<0.05$).

Table Ⅳ. MDA concentrations in non-HDL fraction prior to and after 1, 2 and 3 hours of incubation

MDA equivalents nmol / mg cholesterol	Control	Group H	Group H+E	Group H+C
Baseline	1.6±0.3	1.8±0.5	1.1±0.2[a,c]	1.2±0.5
60[th]min	19.8±3.9	79.8±23.7[a]	16.4±5.4[c]	43.9±9.9[a,c,d]
120[th]min	63.3±6.5	140.8±26.7[a]	27.9±8.2[b,c]	72.6±12.0[c,d]
180[th]min	88.4±11.6	149.3±37.7[a]	36.8±15.1[b,c]	79.7±12.3[c,d]

[a, b] : [a]p<0.01,[b]p<0.001 compared with control group
[c] : p<0.01 compared with Group H
[d] : p<0.05 compared with Group H+E (ANOVA)

DISCUSSION

In recent years, the impact of oxidative stress on the development of many diseases and the preventive

and therapeutic roles of antioxidants have been drawing great deal of attention. In hyperthyroidism, the activity of electron transport chain is increased due to the hypermetabolic state. This increased activity results in accelerated generation of superoxide ions, which are precursors for the production of the hydroxyl radicals which cause lipid peroxidation. Low-density lipoproteins are important target molecules for lipid peroxidation in plasma. While oxidized-lipoproteins are no longer recognized by normal receptors, the scavenger receptors take up these particles in to the cell, triggering excessive mediator release (19-21).

In previous studies the level of plasma MDA, as an indicator of lipid peroxidation, has been shown to increase in hyperthyroidism (17, 22-24). In our study, however, the plasma MDA concentrations in the study groups were not found to be statistically different (Table III).

In the groups receiving L-thyroxine (H, H+E and H+C) serum T_4 and T_3 levels were significantly increased compared with Group C, confirming the establishment of hyperthyroidism. When compared with Groups H and H+E, the serum T_4 level in Group H+C was significantly increased. This could probably explain the lower body weights of Group H+C compared with the other hyperthyroid rats (Table I). However, the T_4 and T_3 levels were significantly lower in Group H+E than in Group H. This finding was consistent with the findings of Seven et al. (22), who concluded that vitamin E could play a preventive role in the development of hyperthyroidism.

The amount of MDA detected in apo B-containing lipoproteins (non-HDL fraction) after incubation with $CuSO_4$ was significantly higher in Group H at the end of the 1^{st}, 2^{nd} and 3^{rd} hours than in Group C. This finding indicates that in hyperthyroidism, lipoproteins containing polyunsaturated fatty acids such as LDL and VLDL readily oxidize when the oxidative stress is enhanced. Compared with Group H, the MDA levels of the non-HDL fraction in Group H+E rats were all significantly lower at the baseline as well as after 1, 2 and 3 hours of incubation with Cu^{2+} (Table IV). In Group H+E, the MDA levels of non-HDL fraction were significantly lower at the baseline ($p<0.01$) and after 2 and 3 hours of incubation than in Group C ($p<0.001$). MDA levels after 1-hour incubation were not significantly different. Vitamin E is the major antioxidant within LDL particles (4, 5). Vitamin E supplementation to hyperthyroid rats has been shown to protect apo B-containing lipoproteins from oxidative damage. On the other hand, compared with Group H, in Group H+C, all readings other than the baseline measurement after incubation revealed significantly lower MDA levels of non-HDL fraction (see Table IV) (this is likely due to the lack of study power). This finding indicates that vitamin C may also play a role in preventing oxidation of apo-B containing lipoproteins. Furthermore, vitamin C as a water-soluble antioxidant may be able to recycle vitamin E (25). In our study, when Group H+E and Group H+C non-HDL fraction MDA levels were compared, all but the baseline measurement were found to be significantly lower in the former group. It is possible that in hyperthyroid rats, the plasma vitamin E levels decrease which in turn, may attenuate the effect of supplemented vitamin C as an antioxidant. After all, only vitamin C was shown to prevent apo B-containing lipoproteins from oxidation. It may be hypothesized that this beneficial effect would be even more pronounced if vitamin E and vitamin C coexisted. Further studies are needed to investigate this point.

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