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Antioxidant Vitamin Levels in Plasma and Low Density Lipoprotein of Obese Girls

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To investigate the antioxidant status of obese children, we analyzed β-carotene and α-tocopherol levels in plasma and low density lipoprotein (LDL). We also analyzed the fatty acid composition of LDL as a substrate for oxidative stress. The plasma β-carotene and α-tocopherol levels were relatively lower in obese girls than in normal controls. However, the plasma α-tocopherol/lipids ratio was significantly lower in obese girls than in normal controls. Both LDL β-carotene and LDL α-tocopherol levels were significantly lower in obese girls than in normal controls, although no obvious differences were observed in plasma levels. In obese girls LDL contained more polyunsaturated fatty acid (PUFA) compared with normal controls. When the peroxidizability Index (PI) was calculated to estimate the susceptibility of lipids to oxidative stress, obese girls had significantly higher PI values than normal controls. Both the LDL β-carotene/PI ratio and the LDL α-tocopherol/PI ratio were significantly lower in obese girls than in normal controls. These results indicate the increased susceptibility of LDL to oxidative stress in obese girls which may promote atherosclerosis later in life.

Keywords: Obese girls, β-carotene, α-tocopherol, LDL, antioxidant, fatty acid

INTRODUCTION

Atherosclerosis is considered to gradually advance in obese people, because of the increased risk of hyperlipidemia and non-insulin dependent diabetes mellitus. Atherosclerotic changes are considered to commence in childhood.[1] Clinical and experimental studies have established that elevated plasma concentrations of LDL are associated with accelerated atherogenesis. Since oxidative modification of LDL is thought to play a role in atherogenicity,[2,3] it is important to assess the susceptibility of LDL of obese people to oxidantive stress.

There are many antioxidant defences against the free radicals generated in biological systems. Beside antioxidant enzymes such as superoxide dismutase, glutathione peroxidase, and catalase, there are various low molecular weight antioxidants like antioxidant vitamins, uric acid, and glutathione in humans. Vitamin E is generally

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accepted to act as a chain-breaking antioxidant in biological membranes and to be protective against free radical-induced injury.[4] Another lipophilic vitamin, β-carotene, a precursor of vitamin A, is an excellent antioxidant for singlet oxygen. These vitamins and ubiquinol are especially important in biomembranes and lipoproteins because of their lipophilic properties. On the basis of the epidemiological evidence that incidence of ischemic cardiovascular disease is inversely correlated with the dietary intake of α-tocopherol and β-carotene,[5] we measured the levels of these antioxidant vitamins in LDL of obese children in the present study.

SUBJECTS

Seventeen obese girls (% obese larger than 20%) who visited Osaka Medical College for health check because of obesity and seven age-matched normal control girls (% obese smaller than 20%) were studied. Obesity was defined using Kato's nomogram, which is a weight-to-height chart commonly used to assess Japanese children.[6] Only girls were enrolled in this study to avoid possible effects of sexural differences. Informed parental consent was obtained for each child. The information of intake frequency of vegetables and fruits was obtained by the questionnaire. All of the normal control girls had no metabolic disorders, no hepatic dysfunction and no excess intake of vegetables. Venous blood was collected into a tube containing EDTA-2Na after an over-night fast. Plasma was obtained by centrifugation for 10 min at 3,000rpm. Plasma total lipids were determined as the sum of the major plasma lipids which are total cholesterol, triglycerides, and phospholipids.

Separation of LDL

Plasma lipoproteins were separated by ultracentrifugation according to the method of Hatch and Lees.[7] Plasma was centrifuged sequentially with adjustment of the density to 1.006–1.063 by NaBr at 4°C using a Hitachi 65P-7 urtracentrifuge with a type RP-65 rotor (Hitachi Koki Co. Ltd. Tokyo, Japan). This 1.006–1.063 fraction was used as LDL in this study. The protein content of LDL was measured by using a Bio Rad Protein Assay kit. The LDL β-carotene and α-tocopherol levels were expressed as nmol/mg LDL protein.

Determination of Plasma and LDL β-Carotene

The plasma and LDL β-carotene concentrations were measured as described previously using HPLC with electrochemical detector (ECD).[8] One milliliter of ethanol containing 0.015% butylated hydroxytoluene was added to 0.2 ml of plasma or 0.2 ml of LDL suspension, followed by vigorous shaking under the nitrogen gas. Then 5 ml of *n*-hexane was added to this mixture, followed by centrifugation at 2,000 rpm at room temperature for 10 min. Next, 4 ml of the hexane layer was evaporated under nitrogen gas. The residue was dissolved in 100 μl of ethanol and a 20 μl aliquot was injected into the HPLC system for the assay of β-carotene by using an Irika Σ871 HPLC apparatus with a reversed-phase C-18 column (Vydac, Hesperia, CA). Detection was done with an Σ871 amperometer (Irika Co, Ltd, Kyoto, Japan). The column was eluted using a solution of methanol/acetonitrile (95:5, v/v) containing 50 mmol NaClO4/l at a flow rate of 1 ml/min. Authentic β-carotene was provided by Nippon Roche Co, Ltd (Tokyo) and was dissolved in ethanol to produce a standard solution, the concentration of which was determined with a Hitachi U-2000 spectrophotometer (Hitachi Co, Ltd, Tokyo), using the absorption coefficient $E^{1\%} = 2620$ at 453 nm in ethanol. We usually employed 500 μg/l of authentic β-carotene in the present study.

Determination of Plasma and LDL α-Tocopherol

Plasma and LDL α-tocopherol concentrations were measured as described previously.[9] One milliliter of 2 mg/ml all-*rac*-tocol in ethanol was

added to 0.2 ml plasma and LDL suspension as an internal standard followed by saponification for 30 min at 70°C with 1 ml of 6% pyrogarol in ethanol and 0.2 ml of 60% KOH in water. After saponification, 2.5 ml of distilled water and 5 ml of *n*-hexane was added, followed by vigorous shaking for 5 min and centrifugation at 3,000 rpm for 5 min to obtain the hexane layer. The hexane layer was subsequently evaporated under nitrogen gas. The residue was dissolved in an appropriate amount of ethanol and then injected into the HPLC system. Assay of α-tocopherol was performed using an Irika P-530 (Irika Instruments, Kyoto, Japan) coupled with an Irika RP-18T ODS column (4 × 250mm). The eluent was methanol/water/$NaClO_4$ (1000:2:7, v/v/w), the flow rate was 1.0 ml/min, and the detector was an Irika Amperometric Σ875.

Determination of Fatty Acid Composition of LDL

Fatty acid methyl esters were prepared by eliminating the extraction step using 2,2'-dimethoxypropane (DMP) as follows.[10] An aliquot of 0.1 ml LDL suspension was placed in a Pyrex tube with one ml of DMP containing 20 μl of concentrated HCl. The tube was flushed with nitrogen and shaken gently for approximately 10 min to facilitate the reaction of DMP with water. Acetone and methanol produced in the reaction and the excess DMP were evaporated under nitrogen gas at room temperature. The residure was mixed with 2.5 ml of the anhydrous HCl-methanol reagent and heated under an atmosphere of nitrogen at 80–85°C in a water bath for 2 h. After the addition of one volume of distilled water, the methyl esters were extracted again with five volumes of *n*-hexane. The extracts were evaporated under nitrogen gas, and dissolved in 50 μl of acetone. The fatty acid composition of the acetone solution of the extract was analyzed by gas chromatography with flame ionization detector (Shimazu GC-8A, Shimazu Corporation, Kyoto, Japan) as described previously.[11]

The peroxidizability index (PI) was calculated as follows to estimate the susceptibility of lipids to oxidative stress.[12]

$$\begin{aligned}PI\ (\%) = {} & (\text{monoenoic}\ \% \times 0.025) + (\text{dienoic}\% \times 1) + \\ & (\text{trienoic}\% \times 2) + (\text{tetraenoic}\% \times 4) + \\ & (\text{pentaenoic}\% \times 6) + (\text{hexaenoic}\% \times 8)\end{aligned}$$

RESULTS

Plasma Antioxidants

Characteristics of study subjects are shown in Table I. Neither age nor height differed between the two groups. In contrast, body weight was relatively higher, and % obese and body mass index (BMI) were significantly higher in obese girls than in normal controls.

Plasma β-carotene level and α-tocopherol were relatively lower in obese children than in normal controls (Table II). There was no significant difference in plasma total lipids between two groups. When the β-carotene/total lipids ratio was calculated, however, it tended to be lower in obese girls than in normal controls. The α-tocopherol/total lipids ratio was significantly lower than in obese girls than in normal controls.

LDL Antioxidants

As shown in Table III, LDL β-carotene was significantly lower in obese girls than in normal controls. Likewise, the LDL α-tocopherol level was significantly lower in obese girls than in normal controls.

TABLE I Characteristics of study subjects

	Normal control	Obese girls
N	7	17
Age (years)	11.3 ± 3.3	10.1 ± 2.1
Height (cm)	138.4 ± 24.0	139.8 ± 11.3
Body weight (kg)	38.8 ± 16.5	47.4 ± 11.6
% Obese (%)	−3.9 ± 11.1	41.7 ± 9.7**
BMI (kg/m^2)	18.2 ± 3.0	23.9 ± 2.3**

Values are the means ± SD.
**$p < 0.05$; compared with normal controls.

TABLE II Plasma β-carotene and α-tocopherol levels in normal controls and obese girls

	Normal controls ($n = 7$)	Obese girls ($n = 17$)
β-Carotene (μmol/l)	0.71 ± 0.20	0.59 ± 0.30
β-Carotene/total lipids (nmol/mg)	0.18 ± 0.06	0.13 ± 0.06
α-Tocopherol (μmol/l)	21.3 ± 5.0	19.5 ± 4.9
α-Tocopherol/total lipids (nmol/mg)	5.33 ± 1.13	4.36 ± 0.78*
Total lipids (mg/dl)	396.4 ± 65.1	438.4 ± 85.6

Values are the means ± SD.
*$p < 0.05$; compared with normal controls.

LDL Fatty Acid Composition

The fatty acid composition of LDL was examined, as shown in Table IV. The LDL of obese girls contained more stearic acid (18:0), oleic acid (18:1), arachidonic acid (20:4), and eicosapentaenoic acid (20:5) compared with that normal controls. The peroxidizability index (PI) value was significantly higher in obese girls than in normal controls.

When the LDL β-carotene/PI ratio and the α-tocopherol/PI ratio were calculated to assess the susceptibility to oxidative stress, the values significantly lower in obese girls than in normal controls.

DISCUSSION

We previously reported low levels of α-tocopherol in the red cells and buccal mucosal cells of obese children, despite relatively higher plasma level.[13,14] When the plasma α-tocopherol level/plasma total lipids ratio, a reliable index of the vitamin E nutritional status[15,16] was calculated in the present study, it was significantly lower in obese girls. These results suggest that obese girls may have poor antioxidant defences in their biomembranes against free radical-induced injury. Plasma β-carotene was relatively lower in obese girls than in normal controls, which is a similar finding of the other report in the adult obese women.[17] When the plasma β-carotene/plasma total lipids ratio was calculated because β-carotene is also a lipophilic vitamin, the ratio is also relatively lower in obese girls like that for α-tocopherol.

It is well known that serum concentrations of carotenoids increase after a large-quantity intake of foods containing large amounts of carotenoids.[18,19] In this study, however, the intake frequency of carotenoids-rich foods was not so different between the obese group and

TABLE III β-Carotene and α-tocopherol levels in LDL of normal controls and obese girls

	Normal controls ($n = 7$)	Obese girls ($n = 17$)
β-Carotene (nmol/mg protein)	0.606 ± 0.249	0.299 ± 0.227**
α-Tocopherol (nmol/mg protein)	13.14 ± 2.73	8.77 ± 1.93**

Values are the means ± SD.
**$p < 0.05$; compared with normal controls.

TABLE IV Fatty acid composition of LDL in normal controls and obese girls

	Normal controls ($n = 7$)	Obese girls ($n = 16$)
C 16:0	30.0 ± 1.4	29.0 ± 2.9
C 18:0	5.8 ± 1.1	7.1 ± 1.3*
C 18:1	16.6 ± 1.8	18.4 ± 1.74*
C 18:2	41.8 ± 3.3	36.4 ± 2.9**
C 20:4	4.1 ± 1.7	5.6 ± 1.5*
C 20:5	0.72 ± 0.4	2.2 ± 1.4**
C 22:6	1.1 ± 0.5	1.40 ± 1.0
PI	71.2 ± 10.2	83.9 ± 16.1*
β-Carotene/PI	8.9 ± 4.7	3.6 ± 2.5**
α-Tocopherol/PI	185 ± 38	110 ± 33**

Values are means ± SD (%).
*$p < 0.05$, **$p < 0.01$; compared to controls.

control. Therefore, the low plasma levels of β-carotene and α-tocopherol in obese children were not explained by their diet. It might be considered that those vitamins are redistributed to the body fat mass, because lipophylic vitamins are stored in adipose tissues.

Since plasma β-carotene and α-tocopherol distributed in lipoproteins[20,21] and are transported in the circulation to the peripheral tissues, the nutritional status of these vitamins should be assessed by their concentrations in the lipoproteins. Steinberg *et al.* have reported that atherosclerosis is related to the oxidation of LDL[2]. Recruitment of circulating monocytes by the chemotactic factors present in oxidized LDL and enhanced uptake of oxidized LDL by resident macrophages may lead to the generation of foam cells. Oxidized LDL is cytotoxic and may cause loss of endothelial integrity. Therefore, the antioxidant vitamins in LDL were measured with analysis of fatty acids as the substrate. As shown in Table III, both β-carotene and α-tocopherol of LDL were significantly lower in the obese girls, although obvious decrease of these vitamins was not found in the plasma levels.

α-Tocopherol exists in the lipid bilayer of biomembranes with a chroman ring on the outside and a side chain inside the membrane. On the other hand, β-carotene exists inside the lipid bilayer of biomembrane due to its hydrophobicity.[22] α-Tocopherol effectively inhibits lipid peroxidation under the air, while β-carotene acts as an antioxidant at low oxygen pressure.[23,24] From these reasons, both vitamins are considered to additionally inhibit LDL peroxidation at their respective locations.

Another factor determining the susceptibility of LDL to oxidant stress is its fatty acid composition. A lower level of linoleic acid in contrast to higher levels of arachidonic and eicosapentaenoic acid were observed in LDL of obese girls than those of control. It might be due to the disturbance of conversion of linoleic to arachidonic acid or utilization of arachidonic acid. Since the rate of peroxidation increases with the degree of unsaturation of lipids, the PI value was calculated in this study (Table IV). There was a significantly higher PI value and a lower ratio of these vitamins to the PI value in obese girls, indicating the antioxidant lack and the increased susceptibility of LDL to oxidant stress. In conclusion, low levels of antioxidant vitamins such as β-carotene and α-tocopherol were found in the LDL of obese girls along with increased peroxidizability of LDL fatty acids. These changes might contribute to the process of atherogenesis later in life if obesity is not controlled. Further study should be required in obese boys, because sexual differences are known in the plasma carotenoid levels.

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