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INGESTION OF EICOSAPENTAENOIC ACID-ETHYL ESTER RENDERS RABBIT LDL LESS SUSCEPTIBLE TO Cu^{2+}-CATALYZED-OXIDATIVE MODIFICATION

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The present study revealed that low density lipoprotein (LDL) obtained from rabbits fed highly purified eicosapentaenoic acid-ethyl ester (EPA-E) (EPA-LDL) was significantly less susceptible to oxidative modification catalyzed by Cu^{2+} as compared to LDL from rabbits fed a normal diet (control LDL). In a comparison of fatty acid composition of LDL, the contents of EPA and docosapentaenoic acid were significantly increased in EPA-LDL as compared to control LDL. The content of arachidonic acid (AA) was the same in both LDL groups. The contents of total cholesterol, free cholesterol, triglyceride, and phospholipid in LDL were unchanged by EPA-E ingestion. These data raise the possibility that EPA may protect LDL from oxidative modification, leading to a reduction of oxidized LDL in atherosclerotic lesions, and that this may be how EPA exert its anti-atherosclerotic action.

In an epidemiological study in Greenland Eskimos it was revealed that the incidence of coronary heart disease was significantly less as compared to that in Danish people despite the Eskimos' high-fat diet [1]. This low incidence of atherosclerotic disease is considered partly related to the action of w-3 fatty acids, such as eicosapentaenoic acid (EPA), which are abundant in marine fish oil [2]. It has been considered that EPA exerts its anti-atherosclerotic action by inhibiting platelet function and correcting

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hyperlipidemia [3,4]. However, the effect of EPA on atherosclerotic tissue changes is not clearly understood. Recently, oxidized low density lipoprotein (Ox-LDL) has been shown to exist in the atherosclerotic vessel wall [5,6], strongly indicating that oxidative modification of LDL is a critical event in the development of atherosclerosis. These data prompted us to investigate whether EPA has any effect on the oxidative modification of LDL. Thus we administered highly purified EPA-ethyl ester (EPA-E) to rabbits and prepared EPA-rich LDL, and we compared the susceptibility of this LDL to the oxidative modification catalyzed by copper ion with that of normal LDL.

Materials and Methods

Reagents

EPA-E was generously donated by Nippon Suisan Co.,Ltd. (Tokyo, Japan). $CuSO_4$, EDTA, and lipidperoxide-test kits were purchased from Wako Pure Chemical Co., Ltd. (Osaka, Japan).

Animals

Six-week-old male Japanese White rabbits were purchased from Clea Japan (Tokyo, Japan) and housed in stainless steel cages. Normal rabbit chow and water were available ad libitum. Rabbits were divided into two groups: one received p.o. ingestion of 300 mg/kg/day EPA-E emulsion (EPA rabbits) and the other was given vehicle alone (control rabbits), for four weeks by way of a rubber stomach catheter without anesthesia.

Preparation of LDL

Rabbits were anesthetized by intravenous injection of pentobarbital and arterial blood was drawn from the carotid artery after a 17-18 hr fast, and the blood was immediately mixed with EDTA (final concentration was 1 mg/ml), as previously reported [7]. LDL (s.g., 1.019-1.063 g/ml) was separated by sequential ultracentrifugation in a Beckman L8-55M equipped with a type 65 rotor using KBr for density adjustment at 105,000xg for 20 hr at 10°C, followed by dialysis overnight against 500 vol of phosphate buffered saline without calcium and magnesium (PBS(-)) at 4°C to remove KBr.

Oxidative modification of LDL

LDL was oxidatively modified according to the method of Kita et al. [8] with minor modifications. Briefly, 1 ml of LDL (250 ug) was mixed with $CuSO_4$ solution to make a final concentration of either 0.5 or 2.0 uM in sterile glass tubes. Then each tube was incubated for various periods up to 72 hr at 37°C in 5% CO_2 and 95% air. At the indicated times, each reaction was terminated by adding a solution of BHT and EDTA to make the final concentrations in the LDL solution 20 uM and 24 uM, respectively, as reported by others [9], and then 100 ul of each LDL solution was sampled for analysis of its lipid peroxide content.

Lipid peroxidation assay

Thiobarbituric acid-reactive substances (TBARS) were determined by the method for the estimation of malondialdehyde (MDA), using the lipidperoxidation-test kit as previously reported [8,9], with minor

modifications according to the assay manual. Briefly, 1.5 ml of 0.9% saline containing LDL (50 ug protein) was mixed with 0.5 ml of 20% TCA and 0.5 ml of TBA reagent (containing 201 mg of thiobarbituric acid in 50% acetic acid) and boiled at 100° C for 60 min. After cooling in water, the mixture was shaken vigorously with 2 ml of n-butanol. After centrifugation at 4000xg for 10 min, fluorescence of the n-butanol phase was measured by spectrophotometer with excitation at 515 nm and emission at 553 nm. Tetramethoxypropane was used as the standard and results were expressed as nmol of MDA equivalent/mg LDL protein.

Determination of fatty acid composition of LDL

Total LDL lipids were extracted with chloroform methanol solution (2/1, V/V) [10] and separated by TLC. Fatty acids were transmethylated, and fatty acid methyl esters were analyzed by gas chromatography equipped with hydrogen flame ionization detector, as previously described [11].

Assay of cholesterol, triglyceride, and phospholipid of LDL

Contents of total and free cholesterol were determined enzymatically by fluorimetry as described by Heider et al. [12]. Contents of triglyceride and phospholipid were assayed enzymatically using Boehringer Mannheim kits. Protein content in LDL was determined by the method of Lowry et al. [13].

Statistical analysis

Statistical significance was determined by Student's t test and each value is given as mean ± SEM.

Results and Discussion

Recently it has been demonstrated that LDL can be oxidatively modified by cells or transition metals, and this Ox-LDL has been shown to be present in atherosclerotic tissues and phagocytized by macrophages through a scavenger receptor [14,15]. Ox-LDL has been revealed to be a potent chemoattractant to monocytes and to exert an inhibitory effect on macrophage motility, it is also cytotoxic to endothelium [5,16]. Hence, Ox-LDL is considered to be one of the most important atherogenic mediators. In the present study we administered EPA-E at a dose of 300 mg/kg/day, an amount equivalent to the daily intake of EPA by Greenland Eskimos [17], to evaluate its effect on oxidative modification of LDL. It was newly demonstrated that LDL from rabbits fed EPA-E became less susceptible to oxidative modification by copper ion as compared to LDL from control rabbits (Fig.1). In addition EPA content was significantly increased in LDL from EPA rabbits (Fig.2). In contrast, the contents of arachidonic acid, cholesterol, triglyceride, and phospholipid were the same level as in both EPA-rich LDL and control LDL (Fig.3). It was previously reported that

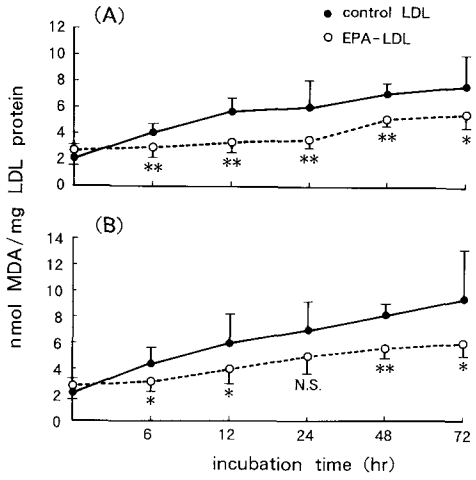

Fig.1. Effect of EPA-E ingestion on oxidative modification catalyzed by copper ion. EPA-E (300 mg/kg/day) or vehicle alone was administered for four weeks through a stomach catheter. LDL (250 μg/ml) was mixed with final concentration of either 0.5 μM (A) or 2.0 μM (B) of $CuSO_4$ for the indicated periods. TBARS in the LDL solution were determined as described in "Materials and Methods." Each point represents mean±SD of seven animals. *: p<0.05, **: P<0.01, N.S.: not significant.

the generation of TBARS, produced during copper ion-catalyzed LDL oxidation, paralleled the macrophage uptake of LDL [18]. Thus the inhibitory effect of EPA-E seems to be quite important with respect to the prevention of atherosclerotic tissue changes.

The precise mechanisms of the inhibitory effect of EPA are still unclear. Of great interest was the finding by Steinbrecher et al. [18],

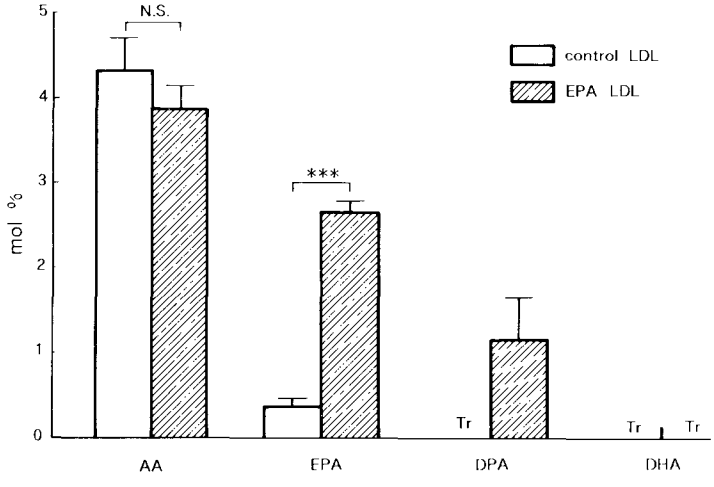

Fig.2. Effect of EPA-E ingestion on fatty acid composition in LDL. Each data is given as mean±SD of seven animals.
***: p<0.01, n.s.: not significant, Tr: trace amount.

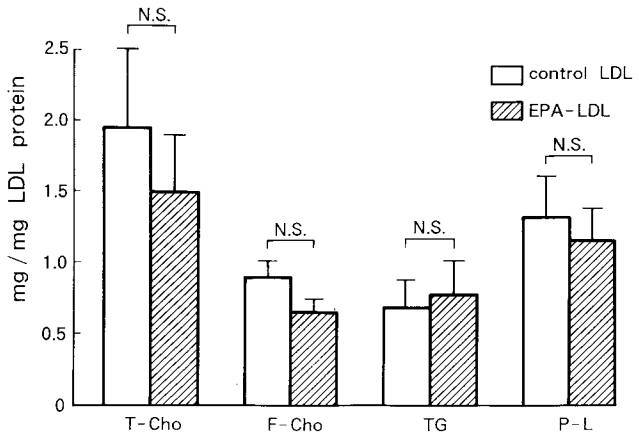

Fig.3. Effect of EPA-E ingestion on the contents of cholesterol, triglyceride, and phospholipid in LDL. Each data is given as mean±SD of seven animals. No significant differences were observed between EPA-rich LDL and control LDL.

who reported that modification of LDL by copper ion includes the degradation of phosphatidylcholine and the production of lysophosphatidylcholine, suggesting an involvement of a phospholipase A_2 (PLA_2) activity intrinsic to LDL. Previously it was suggested that EPA, which was incorporated into membrane phospholipids, had an inhibitory action on membrane bound PLA_2 [19]. Therefore, one possibility is that EPA might exert its effect by influencing PLA_2 activity in LDL. It should be considered that LDL contains several kinds of anti-oxidants, such as tocopherols, b-carotene, lycopine, and retinyl stearate. It was reported that when these substances were consumed, LDL became susceptible to oxidative modification [20,21,22,23]. Interestingly, Croset et al. [24] reported that the ingestion of purified EPA significantly increased the content of tocopherol in platelets. And the low content of platelet tocopherol may be linked to an increased susceptibility of platelets to lipid peroxidation [25]. Thus other possibility is that EPA-E ingestion may influence the contents of radical scavengers in LDL. The present study further revealed that the content of docosapentaenoic acid (DPA) was also significantly increased in EPA-rich LDL as compared to that in control LDL (Fig. 2). Therefore, further study should be done to elucidate the effect of DPA on oxidative modification of LDL.

Recently it was demonstrated that probucol has an anti-atherogenic action and that this reagent exerts an inhibitory effect on oxidative modification of LDL catalyzed by copper ion [8]. Contrary to probucol, as EPA is consumed in the normal human diet, we can expect more physiologic action in the prevention of atherosclerosis in concert with its beneficial effect on platelet function and serum lipids. The present study raises the possibility that EPA may protect LDL from oxidative modification in the vessel walls and that the anti-atherogenic action of EPA can be partly ascribed to this action. But it still remains to be elucidated whether EPA-rich LDL has the same characteristic in in vivo cellular modification.

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