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Fatty Acid Composition and Functional Propreties of Low Density Lipoprotein and Oxidized LDL from Human Plasma

Jae-Hoon Choi, Hyun-Mi Cho, Heung-Soo Son* and Tae-Woong Kim†

Dept. of Biochemistry, Kangwon National University, Chuncheon 200- 701, Korea
**Korea Nutrition Institute, Hallym University, Chuncheon 200- 702, Korea*

Abstract

Human plasma low density lipoprotein (LDL) is the major factor of coronary heart disease. But recent studies suggest the normal LDL can be readily oxidized by oxygen free radicals and not interact with LDL receptors. Lipoprotein particles consist of lipid and protein, and fatty acids are prone to oxidation. The fatty acid compositions of LDL from Koreans was compared with that of Westerners. From the results, the ratio of unsaturated fatty acids of Korean and Westerner approximately 30 and 70%, respectively, which means Westerners are more labile in the lipid oxidation of LDL than Koreans. Normal LDL was incubated with $CuSO_4$ in PBS to lead for the peroxidation of LDL, and it was tested by the detection of TBARS and free radicals. Then, ascorbate, α-tocopherol and hyaluronic acid were found to have effects of antioxidants on LDL oxidation. The amount of free radical increased as the extent of oxidation increased. The time course of free radical formation was similar to TBARS. Therefore, determination of free radical by luminometer was much more convenient than that of TBARS.

Key words : fatty acid composition, human plasma, LDL, oxidized LDL, free radical, TBARS (Thiobarbituric acid reacting substance)

INTRODUCTION

Elevated levels of plasma low density lipoprotein (LDL) correlate directly with a risk of coronary heart disease. LDL is a main carrier for cholesterol in the blood stream, and it is well established that cholesterol deposits in the arteries stem primarily from LDL and that increased levels of plasma LDL correlated with an increased risk of atherosclerosis[1,2]. LDL particles are incoporated into tissue by way of a cell-surface receptor that recognizes LDL's apoprotein component, results cholesterol synthesis regulated at liver[3]. The initial suspicion was that this LDL receptor contributes both to the normal uptake to LDL and to the abnormal deposition of LDL cholesterol within artery walls.

In 1979, Goldstein discovered that macrophages ordinary indifferent to LDL, have scavenger receptor, by which they can recognize and ingest a modified LDL. Initial studies involved in LDL particles acetylated *in vitro*; subsequent research showed that scavenger receptors also bind oxidized LDL-a modification more likely to occur *in vivo*[4,5]. Various lines of research provide strong but not conclusive evidence that LDL may become oxidized *in vivo* and that the oxidized LDL is the species involved in the formation of early atherosclerotic lesions. Early atherosclerotic lesion is characterized by massive accumulation of cell filled with lipid droplets consisting of cholesterol and cholesteryl esters. Because of their foamy appearance such cells are called foam cells[6~8].

Several studies carried out oxidative modification of LDL by endothelial cells and Cu^{2+} *in vitro*, were shown that copper-mediated oxidation of LDL noted that the oxidized LDL by copper had properties similar to endothelial cell-mediated oxidation of LDL that differs in the several ways compared to the native LDL[9~12].

Not all fatty acids are equally prone to oxidation. Polyunsaturated fats are actually more vulnerable to oxidation than saturated fats[13]. Among the polyunsat-

† To whom all correspondence should be addressed

urated fats, arachidonic acid is of particular interest. A key byproduct of arachidonic acid's oxidation is malondialdehyde (MDA). MDA can directly alter the conformation of apo B-100[14].

The present studies were undertaken to investigate the fatty acid composition of LDL and oxidized LDL, first. Secondly, functional changes of oxidized LDL and properties between mildly and extensively oxidized LDL were tested.

MATERIALS AND METHODS

Materials

2-thiobarbituric acid (TBA), trypsin inhibitor, aprotinin, 2,4,6-trinitro benzen sulfonic acid (TNBS) and 1,1,3,3-tetramethoxypropane (TMP) were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). Barbital sodium was purchased from Junsei Chemical Co. (Tokyo, Japan). Trichloroacetic acid (TCA) was obtained from Janssen Chimica (Geel, Belgium). All chemical reagents were purchased from J. T. Becker (Philipsberg, NJ, USA).

Isolation of low density lipoproteins

The blood from healthy donors was collected to a sterilized bag contained 0.2M EDTA, 2.5% sodium azide, 2000U/ml aprotinin and 0.3M NaCl. The plasma was obtained by centrifugation of the fresh blood at 3000rpm for 30min at 4°C. Aprotinin (0.055 Units-/ml), 0.05% EDTA and 0.05% NaN_3 were added to the plasma to prevent for coagulation and further hydrolysis. The LDL, $1.025 < d < 1.055$g/ml, were isolated by sequential ultracentrifugation using a KBr gradient at 40,000rpm for 15 hrs at 4°C with a Beckmam 50.2 Ti rotor, followed by one more runs for 24 hrs under the same conditions[15]. The protein concentration of the LDL was determined by the method of modified Lowry *et al.*[16]. Purified LDL was dialyzed against 0.1M ammonium bicarbonate (NH_4HCO_3), pH 8.0 buffer with dialysis bag (Spectra/No.2). The purification of LDL was identified by 5-14% SDS-PAGE.

Delipidation of LDL

The LDL (50mg) were lyophilized and subjected to two extractions with 50ml of ether/ethanol (3 : 1, v/v)[17]. At each step of the extraction, the protein- solvent mixture was incubated at -20°C for 1h and pelleted by low speed centrifugation (2,000 × g for 20 min) and the solvent was removed by aspiration. After the final ether-ethanol extraction, the protein (apo B) was washed once with anhydrous ether and pelleted, and the solvent was removed by aspiration.

Gas chromatography

The analysis of fatty acid composition was performed using gas chromatography (Hitachi model 163) equipped with F. I. detector. A 2m × 3mm (ID) glass column containing GP 3% SP-2310/2% SP-2300 on 100/120 chromosorb was used for the GLC analysis[18]. The temperature of injector was 220°C. The flow rate was : nitrogen, 39ml/min : hydrogen, 36 ml/-min : and air, 500ml/min. Chart speed was 0.5 cm/-min.

Oxidation of LDL

IAF-LDL was incubated with cupric sulfate (5μM) at a concentration of 400g/ml protein in 1ml PBS in the presence and absence of antioxidants (40μM ascorbic acid, 40μM α-tocopherol, and 10nM hyaluronic acid) at 37°C for each times. The extent of lipid peroxidation was measured as thiobarturic acid reactive substances (TBARS) and expressed as MDA equivalents[14, 16]. Briefly, 1.0ml of 25% trichloroacetic acid-(TCA) was added to 0.5ml samples, followed by 1.0 ml of 1% TBA. The samples were vortexed and incubated at 95°C for 50min, then they were centrifuged-(1000 × g) for 15 min. Supernatant TBARS were detected by UV-spectrophotometer at 532nm. Samples of freshly diluted 1,1,3,3-tetramethoxypropan (TMP) were used as standards.

Determination of free radical

Amounts of radical were determined by using 6 channel Biolumat LB 9505 C (Berthold Co., Germany). 0.2 mg/0.5ml of oxidized LDL was loaded in the tube and personal computer was used to record the changes of free radicals of each samples by using the LB 9505 C program[4].

Determination of TBARS

Lipid peroxidation was estimated by measuring the amount of thiobarbituric acid (TBA) reactive materials at 532nm that were generated.

RESULTS AND DISCUSSION

Isolation of LDL

The yield of plasma from total blood was approximately 57.1% and pure LDL was obtained 4.2% compared to the original amount (Table 1). LDL-containing fractions obtained by sequential ultracentrifugation were dark yellow. Apo B-100 is the apoprotein of LDL, and the protein concentration of pure LDL was 13.2mg/ml when protein concentration was quantitated at 280nm. SDS-polyacrylamide gel electrophoresis showed the only one peak which means apo B-100 was pure and other apoproteins were not contaminated during the isolation (Fig. 1).

Comparison of fatty acids

Fatty acid compositions of five LDL samples from Koreans were analyzed using gas chromatography with F. I. detector for the comparison of Koreans and Westerners, but arachidonic acid (20 : 4), particularly interested fatty acid, was not detected (Table 2). The extracted lipid-moiety was separated into two subclasses, first and second extraction. The major fatty acids were palmitic (16 : 0), stearic (18 : 0), oleic (18 : 1) and linoleic acid (18 : 2) (Fig. 2). The fatty acid composition of westerners was provided from Huang *et al.*[19] Major fatty acids (16 : 0, 18 : 0, 18 : 1 and 18 : 2) ratios of Koreans and Westerners were approximately 75.1% and 85%, respectively. Also, composition of unsaturated fatty acids, which are more vulnerable to lipid peroxidation, was significa-

Table 1. Purification of LDL from human blood

Step	Volume	Yield (Total volume)	LDL concentration (Total volume concentration)
Blood	350ml	100%	–
Plasma	200ml	57.1%	–
After 1st centrifuge (HDL, LDL, Albumin)	120ml	34.3%	–
After 2nd centrifuge (LDL)	15ml	4.2%	13.2 mg/ml

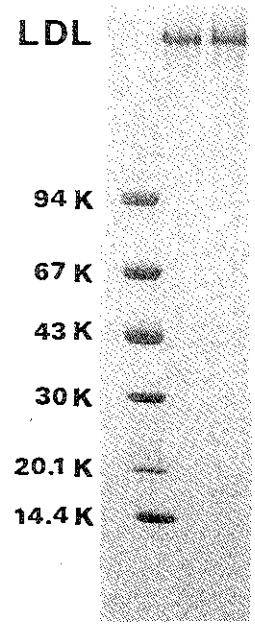

Fig. 1. SDS-PAGE pattern in 5~14% gradient slab gel.

Table 2. A comparison of fatty acid composition between Korean and Westerners

Korean†			Westerners*		
Fatty acid	1st Extraction	2nd Extraction	Fatty acid	1st Extraction	2nd Extraction
14 : 0	4.98%	3.31%	16 : 0	11.31%	40.63%
16 : 0	12.58%	20.15%	16 : 1	2.37%	0.73%
16 : 1	4.71%	8.10%	18 : 0	5.40%	6.79%
18 : 0	16.13%	16.33%	18 : 1	20.15%	12.73%
18 : 1	20.57%	14.55%	18 : 2	48.06%	32.39%
18 : 2	11.49%	21.23%	others	12.81%	6.73%
Others	18.44%	16.23%			
Saturated FA	16.71%	47.42%	Saturated FA	16.71%	47.42%
Unsaturated FA	70.48%	45.85%	Unsaturated FA	70.48%	45.85%

†LDL from Koreans subjected to two extractions with ether-ethanol(3 : 1, v/v). Each step of extraction was analyzed by GLC
*Fatty acid composition from Westerners are from Huang *et al.*[19]

ntly different, 31.9% and 70.5% of Koreans and Westerners, respectively.

From the results, Koreans, compared with Westerners, are less affective in LDL peroxidation. Unsaturated fatty acid composition of Koreans was 31.9%, while that of Westerners is 70.5% at the first extract moiety. Not all fatty acids are equally prone to oxidation. Polyunsaturated fats, although less closely associated with coronary heart disease than saturated fats, are actually more vulnerable to oxidation. At present, dietary intake of polyunsaturated fats are recommend to reduce heart disease, but current research into the role of polyunsaturates vs monounsaturates in atherogenesis bears close scrutiny.

Measurement of TBARS

Fig. 3 shows the TBARS formation of each oxidized LDL. LDL was readily oxidized when incubated with PBS in the presence of cupric sulfate. Incubation of LDL (400g/ml protein) with copper resulted in the generation of TBARS that were approximately 6-fold higher in magnitude than that generated by unincubated LDL. The amount of TBARS was 59.8 nmol/mg LDL after 24 hrs of LDL oxidation by 5μM of copper. A similar result was reported from Parthasarathy *et al.*[20] that by the same condition of copper.

On the incubation in the presence of ascorbic acid and α-tocopherol, LDL under similar conditions also produced increased TBARS, However, the amounts of TBARS produced were much lower as compared to those produced when LDL was incubated with copper in the absence of antioxidant. But, antioxidative effects of α-tocopherol decreased after 12 hrs of oxidation. A previous study[11] reported that α-tocopherolol had an inhibitory effect on the oxidative modification of LDL only for 5hr. When α-tocophrol was added, the amounts of generated TBARS were two times higher than those of ascobic acid at 24hr. On the other hand, hyaluronic acid showed decreased formation of TBARS compared to ox-LDL, although that was higher in TBARS than ascorbic acid and α-tocopherol added groups. This antioxidative effect could be due to the decrease of free radical formation during lipid peroxidation by copper.

In the course of lipid metabolism, circulating LDL particles migrate across the endothelial border and enter the arterial subendothelial space. The subendothelium is more favorable environment for lipid oxidation than circulating plasma, because natural antioxidants are abundant in plasma[8]. And, various antioxidant agents, such as superoxide dismutase and carotene, vitamin C and vitamin E are also present[9].

Determination of free radical

Measurements of TBARS was a time-consuming and not-reproducible test, because TBA is an unspecific reactant to malondialdehyde[14]. Therefore, to im-

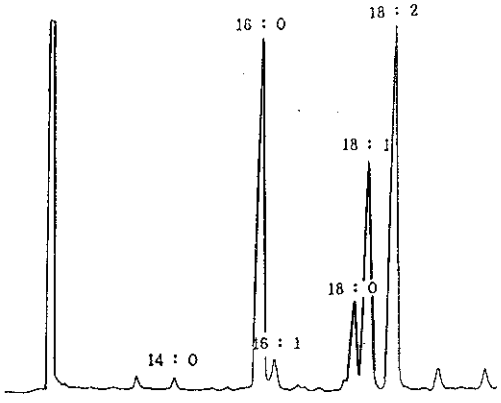

Fig. 2. Fatty acid pattern of LDL ewtracted with ether/ethanol(3 : 1, v/v) by GLC.

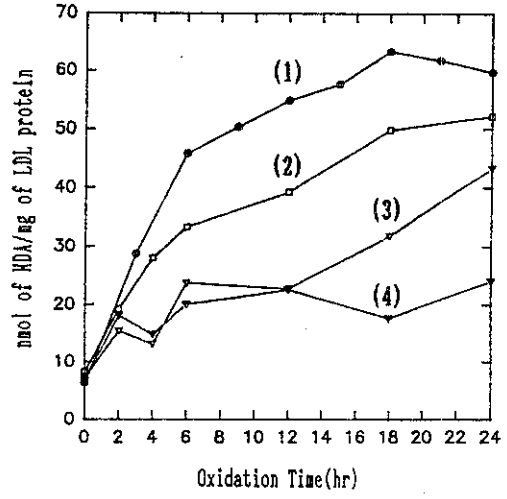

Fig. 3. Thiobabituric acid reacting substance (TBARS) of oxidized LDL with and without addition of antioxidant.
(1) Ox-LDL (2) Ox-LDL + Hyaluronic acid
(3) Ox-LDL + Vit. E (4) Ox-LDL + Vit. C

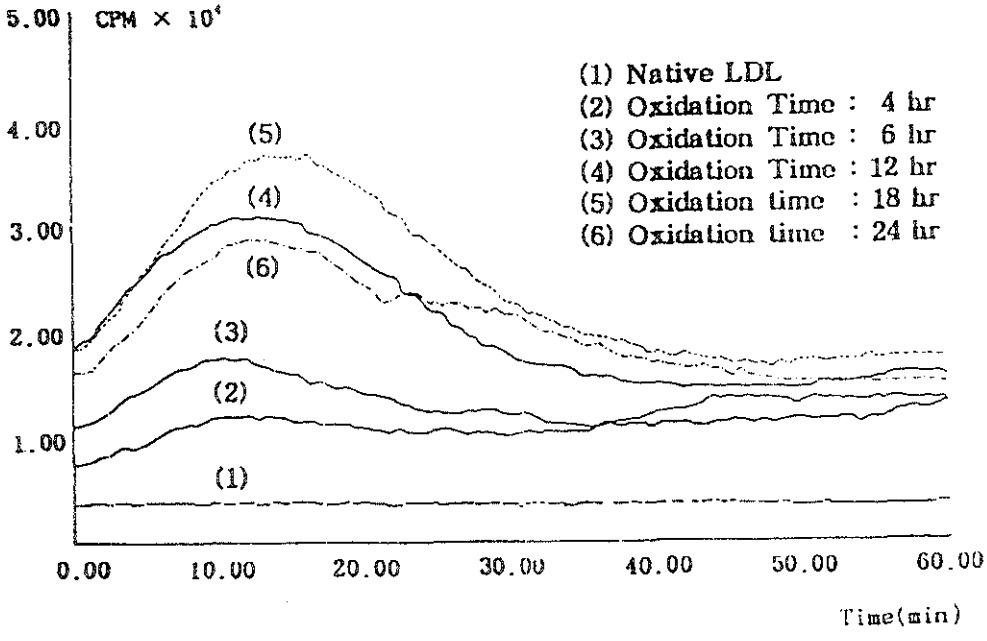

Fig. 4. Time course of free radical formation of oxidized-LDL by luminometer.

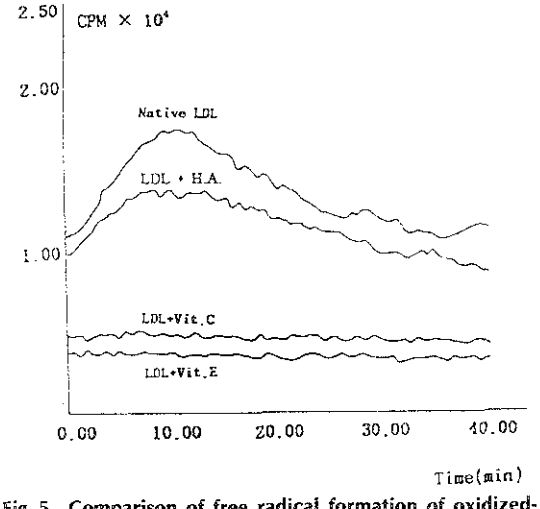

Fig. 5. Comparison of free radical formation of oxidized-LDL in presence and absence of antioxidant.
ox-LDL means LDL was oxidized for 6hrs.

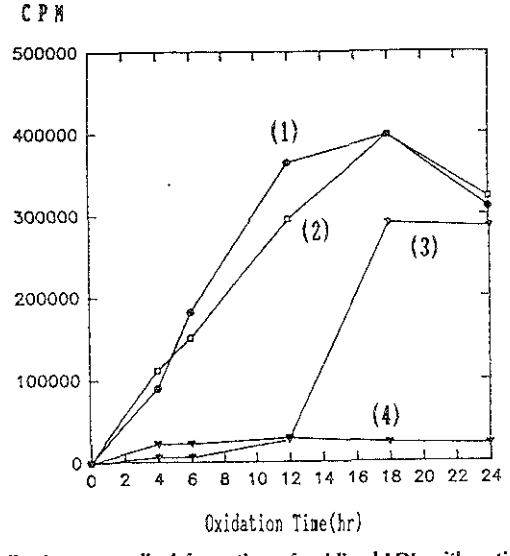

Fig. 6. Free radical foramtion of oxidized-LDL with antioxidant by luminometer.
(1) Ox-LDL (2) Ox-LDL + Hyaluronic acid
(3) Ox-LDL + Vit. E (4) Ox-LDL + Vit. C

prove this study, the amounts of free radical were determined during the copper mediated LDL oxidation using an instrument, Luminometer (Fig. 4, 5, 6). In time coure of free radicals produced during the LDL oxidation, the overall pattern (Fig. 6) was similar to TBARS (Fig. 3).

Oxidized LDL in the absence of ascobic acid or α-tocopherol generated approximately 4-fold higher in amounts of free radicals than that generated in the presence of them until 12 hr, while, generated amounts of free radicals in the absence of antioxidants decreased after 18 hr. On the other hand, formed free radicals by LDL incubated with α-tocopherol and copper were increased significantly after 12 hr. From

this results, we can conclude that α-tocopherol was a less effective antioxidant than ascorbic acid in lipid peroxidation of LDL. However, hyaluronic acid was not effective to reduce the formation of free radicals during the LDL oxidation. Also, comparison of the time course of free radicals of ox-LDL in the presence and absence of antioxidants was shown on Fig. 5. From the results, determination of free radical formation was much more effective than measurement of TBA-RS for degree of LDL oxidation by an instrumental method.

Normal LDL concentration can be controled by diet but oxidation of LDL cannot because it is caused by exogenous conditions, aging, nitrogenous products of tobacco smoke or airpollution, and such compounds as carbon tetrachloride and bleomycin. Therefore, the way to effective retardation of LDL oxidation rate is intake of antioxidants.

In conclusion, ox-LDL altered its biological properties and was a greater risk factor to atherosclerosis than the native LDL.

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인체 혈장에서 분리한 LDL과 LDL의 지방산 조성과 기능성의 변화

최재훈 · 조현미 · 손홍수* · 김태웅†
강원대학교 생화학과
*한림대학교 한국영양연구소

요 약

인체의 혈장 저밀도 지단백(LDL)은 관상동맥경화 발병의 주 요인이다. 그러나 최근의 연구들은, 정상적인 LDL은 산소 자유라디칼에 의해 쉽게 산화되며, 결과 LDL 수용체와 결합하지 못한다고 밝히고 있다. 따라서 이 변형된 형태의 산화된 LDL은 macrophage scavenger receptor에 의해 인식되어 foam cell을 형성하여, 동맥혈관이 좁아지는 역할을 수행한다고 알려지고 있다. 지질과 단백질로 구성되어 있는 LDL의 산화에는 실제 작용성을 가진 단백질보다는 지질이 절대적인 작용을 한다. 지질의 산화에는 지방산이 중요한 작용을 하므로, 한국인의 LDL의 지방산 조성을 분석하여 서양인과 비교하였다. 결과, 한국인의 불포화 지방산의 비율이 총 지방산 함량의 약 30%인 반면 서양인은 약 70%의 분포를 갖고 있는 것으로 발표되었다. 따라서 한국인이 서양인에 비해 LDL의 산화에 대한 영향을 적게받을 수 있으며, 따라서 동맥경화나 심장병의 발생률이 훨씬 적을 것으로 결론을 내릴 수 있다. 정상적인 LDL을 황산구리와 함께 배양하여, 지방의 산화를 유도 하였으며 이의 정도를 지방산 산화의 생성물인 TBARS를 측정하여, LDL이 산화될 때 생성되는 자유라디칼의 양을 측정하므로서 비교하였다. 이 때, 항산화제인 비타민 C; 비타민 E와 히알우로닉산을 첨가하면 LDL의 산화가 억제되는 효과를 확인하였다. 자유 라디탈이 증가함에 따라 산화의 정도도 증가하였으며, 자유라디칼 형성의 경시적 변화는 TBARS와 유사하였다. 따라서 luminometer에 의한 자유라디칼의 정량은 TABRS에 의한 것보다 훨씬 간편한 것으로 나타났다.