EFFECT OF ∝-TOCOPHEROL AND BUTYLATED HYDROXY ANISOL (BHA) IN CU^{2+} INDUCED OXIDIZED LOW DENSITY LIPOPROTEIN

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Summary

The increased levels of low-density lipoprotein (LDL) are directly related to the development of atherosclerosis and oxidation of LDL induced by Cu^{2+} caused marked peroxidation as shown by increase in the levels of lipid peroxide by 10.79 folds. Addition of various concentrations of ∝-tocopherol and BHA showed concentration dependant inhibition against the formation of TBARS which increased by 18.40 fold as compared to native LDL, inhibited by 88%.

The oxidation of LDL induced by Cu^{2+} caused increase in REM of this lipoprotein upto 33% and addition of various concentration of ∝-tocopherol or BHA reversed significantly the REM of oxidized LDL to a maximum of 17% and 21% respectively at their highest concentration. Hence Cu^{2+} induced charges in LDL, lipids and proteins were very marked. So the formation of lipid peroxide and fragmentation of LDL, apoprotein was significantly inhibited by ∝-tocopherol or BHA in vitro.

Key Words : Atherosclerosis, ∝-tocopherol , Apolipoprotein

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Introduction

The changes that occur in the arterial wall in the course of atherosclerosis are probably a reaction to injury to the endothelium, which may result in either loss of the living cells or their dysfunction (1). The injury as such could be mediated by infections (2), ageing (2,3), mechanical disturbances in blood flow or chemical and environmental intoxications (1,4).

The marker of disorder of lipid metabolism is the increased level of plasma cholesterol. Cholesterol is transported in the shape of water soluble complexes called as low density lipoprotein (LDL). The increased levels of LDL are directly related to the development of atherosclerosis. Various chemical modifications such as oxidative, acetylated and glycolated forms of LDL have been found in pathogenesis.

Recently much attention has been focused on the role of modified low density lipoprotein (LDL) in the initiation and propagation of athersclerosis (7). The modification in LDL alters its biological properties which in turn accelerate endocytosis and cholesterol accumulation in arterial walls by macrophage through it's scavenger receptor.

It is now well established that free radicals play an important role for the initiation and progression of metabolic disease. Oxidation of LDL changes its capacity to interact with cells due to alterations in the recognition mechanism used for internalization into cells and it is potential atherogenic effect depends largely on the extent of oxidation (13). Oxidation of LDL can be induced by incubation with cells in culture or by incubation with a heavy metal ions such as copper or iron. Modified forms of LDL is associated with changes in the LDL particles such as formation of lipid peroxides, fragmentation of apolipoprotein B-100 (apo B) and also changed in the electronegative charges on LDL after oxidation was assessed by agarose gel electrophoresis.

Materials and Methods

I. Polyionic precipitation of serum lipoproteins

Normal human healthy blood was collected after 3 hrs at room temperature, the blood was centrifuged at 4000rpm in cold. The serum was mixed with one tenth volume of heparin and manganese chloride ($MnCl_2$) and incubated at 40°C-45°C for 90 minutes. The incubation mixture was centrifuged at 8000rpm for 30 minutes at 20°C, VLDL complex was separated and resuspended in sodium bicarbonate and sodium chloride solution at 0°C. The clear solution of VLDL was mixed with barium chloride and sodium chloride solution of VLDL free from heparin and $MnCl_2$.

The supernatant was further precipitated for LDL by mixing with solution for dextran sulphate and $MnCl_2$ with a final concentration of 0.013% at 0.025M respectively. This reaction mixture was kept at 0°C for sixteen hours before centrifugation as above. The precipitate containing LDL was purified by suspending with barium chloride solution and centrifuged supernatant free from VLDL or LDL was further precipitated. To get HDL this supernatant was mixed with dextran sulphate and $MnCl_2$ solution to attain the final concentration of 0.65% and 0.2M respectively in reaction mixture and centrifuged at 10,000rpm for 15 min. at 0°C. The HDL precipitate was taken out from lower supernatant and dissolved with 2% sodium citrate in 1% aqueous NaCl at 4°C for 16hrs and centrifuged as above. This HDL solution including the solution of VLDL, LDL and lower supernatant, were dialyzed against 0.1M aqueous NaCl for 36 hrs at 4°C.

II. Oxidation of LDL in Vitro

The reaction mixture containing 0.25 ml LDL, 0.25 ml $CuCl_2$ (10 μmole/ml reaction mixture) added without or with different concentrations of ∝- tocopherol or BHA in phosphate saline buffer to a final volume of 1 ml. Separate tubes containing LDL but not ∝-tocopherol or BHA as well as reference tubes containing respective amounts of ∝-tocopherol and BHA but not LDL were prepared, after incubation at 37^oC for sixteen hours, 100μl volume was taken out from each experimental tube to check the changes in

the protein moiety by relative electrophoretic mobility pattern an agarose gel electrophoresis . Remaining volumes of the tubes were delipidated with $CHCl_3$:CH_3OH at 40^o-50^oC and filtered. The lipid extracts were dried under the stream of nitrogen gas and used for the estimation of total lipids, lipid peroxide and lipid hydroperoxides.

1. Cholesterol was estimated by the method of Zlatkis et al (1957) (14)
2. Total lipid content was estimated by the method of Zollner et al. (15)
3. Lipid peroxide was estimated by the method of Ohkawa et al. (16)
4. The apoprotein was precipitated by as Redding et al. (17)

Statistical Analysis : Data was analyzed using student's 't' test and standard deviation (SD) was calculated by the formula.

Electrophoresis of Lipoprotein was performed by Agarose 0.5 & 1 % gel

Pre-staining of LDL samples: 60 mg of Sudan black was dissolved in 5 ml of poly ethylene glycol containing 0.01 ml Tween-80 and filtered. 100 µl of LDL samples and 50 µl sudan solution were mixed with sufficient amount of sucrose to make it viscous.
The apparatus was allowed to run between 90-110 volts for 4 to 12 hrs and after electrophoresis REM is measured and compared with native LDL (Unoxidized).

Electrophoresis of Agarose –Acrylamide composite gel was performed in different ratio i.e 1:1, 2:1 & 1:3 ratio. These gel plates were seen at 120V for 4 hrs. The relative electrophoretic mobility (REM) was determined as compared to mobility of native LDL (unoxidised) from the point of application on gel.

Results

Table 1: Oxidation of low-density lipoprotein

Experimental Schedule	Total Lipid	Apoprotein	Total Cholesterol
Native LDL	2.58±0.40	0.69±0.05	1.54±0.40
Oxidised LDL	2.23±0.46 (14)	0.62±0.107 (10)	1.40±0.20 (9)
Oxidised LDL in presence of Cu^{2+}	1.02±0.06 (60)	0.32±0.05 (53)	0.77±0.09 (50)

Values expressed as mg/ml LDL solution are mean ± SD of two separate observations. Values in parenthesis are percent decrease as compared to native LDL. 1 ml solution contains 3.5 mg of LDL.

Table 2: Effect of ∝-tocopherol on Cu^{2+} induced lipid peroxidation of LDL

Experimental Schedule	**Lipid Peroxide Nmol MDA/mg protein**
Native LDL	2.78±0.6
Oxidised LDL	30.02±2.4
Oxidation of LDL in presence Of ∝-tocopherol(μmol)	
0.05	28.03±3.8 (7)
0.1	18.27±2.0 (39)
0.2	10.24±1.2 (66)
0.3	6.60±0.8 (78)
0.4	6.06±0.3 (80)
0.5	4.2±0.2 (86)

Values are mean ± SD of two separate observations

Table 3: Effect of butylated hydroxy anisol (BHA) on Cu^{2+} induced LDL oxidation

Experimental Schedule	**Lipid Peroxide Nmol MDA/mg protein**
Native LDL	3.09±0.90
Oxidised LDL	56.86±4.0
Oxidation of LDL in presence Of BHA (μmol)	
0.1	47.82±2.8 (16)
0.2	26.20±1.8 (54)
0.3	15.40±1.6 (73)
0.4	13.42±1.5 (76)
0.5	6.77±0.4 (88)

Values are mean ± SD of two separate observations.

Table 4: Relative electrophoretic mobility (REM) pattern of LDL

S.No	Experimental Schedule	Mobility	Relative Electrophoretic mobility (REM)
1	Native LDL	0.90	100
2	Oxidised LDL by Cu^{2+}	1.20	133 (33%)
3	Oxidised LDL by Cu^{2+} In presence of ∝-tocopherol 0.1μM 0.25μM 0.5μM	 1.15 1.10 1.00	 127 122 111 (17%)
4	Oxidised LDL by Cu^{2+} In presence of BHA 0.1μM 0.25μM 0.5μM	 1.12 1.10 0.95	 124 122 105 (21%)

Values are mean of two separate observations.

Data in Table 1 showed that during aerobic oxidation, there was depletion of total lipid, total cholesterol and apoprotein components of LDL by 10-22%. These changes were more pronounced (60%). The inhibitory action of ∝-tocopherol and BHA was studied as shown in Table 2 and 3. The oxidation of LDL induced by Cu^{2+} caused marked peroxidation as shown by increase in the levels of lipid peroxide (TBARS) by 10.79 folds. Addition of various concentration of ∝-tocopherol (0.05-0.5 μM) showed concentration dependent inhibition against the formation TBARS. At 0.5 μM of ∝-tocopherol, the protection observed was 86% (Table-2). Similarly BHA at 0.05 μM concentration in above reaction mixture, in which TBARS was increased by 18.40 fold as compared to native LDL, inhibited the oxidation by 88% (Table 3). Table 4 showed that oxidation of LDL caused increase in REM of this lipoprotein added with Cu^{2+} (33%). Addition of various concentrations of ∝-tocopherol (0.1-0.5 μM) or BHA (0.01-0.05 μM) reversed significantly the REM of oxidized LDL to a maximum by 17% and 21% respectively, at their highest concentrations.

Discussion

Our data showed that LDL is highly prone to oxidative changes in its lipid as well as apoprotein parts. Increased oxidation of LDL-lipids by Cu^{2+} may be due to nonenzymatic stimulation of the reaction of peroxidation mediated through free radical generation. During peroxidation LDL had to undergo the degradation of lipids as well as protein. The lipid peroxidation products; TBARS, has been observed as a measure to check the extent of lipid peroxidation. The addition of ∝-tocopherol or BHA significantly inhibited the formation of TBARS. BHA was more active than ∝-tocopherol. However, BHA cannot be used as LDL protective drug. Whereas ∝-tocopherol is a biological antioxidant.

It has been suggested that increasing dietary uptake of this vitamin, protected LDL against oxidation. Oxidation of LDL caused the fragmentation of APO-100 into B-48 and make this lipoprotein more electronegative as evidenced by the increased REM of oxidized LDL on agarose gel. The high density lipoprotein (HDL) in body plays a very important role as an antioxidant to neutralize the free radical reactions for LDL oxidation. Carvadilol, probucol, the known antihypertensive and lipid lowering agent also, by part play the role of an antioxidation for the protection of LDL.

Hence we conclude that Cu^{2+} induced charges in LDL lipids and protein were very marked. The lipid peroxidation formation and fragmentation of LDL, apoprotein was significantly inhibited by ∝-tocopherol, or BHA in vitro.

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