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Effect of ursodeoxycholic acid on copper induced oxidation of low density lipoprotein

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Received January 16, 2004, accepted October 8, 2004

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Pharmazie 60: 609–613 (2005)

The aim of this study was to investigate copper (Cu^{++}) induced oxidation state of LDL isolated from obstructive jaundice (OBJ) patients with hyperlipidemia and the effect of UDCA on the same. LDL was isolated and oxidation was induced by 5mM $CuSO_4$ with/without UDCA at different concentrations. LDL oxidation was assessed at different time intervals in terms of conjugated dienes, hydroperoxides and 'thiobarbituric acid reacting substances' (TBARS). The change in the level of endogenous LDL α-tocopherol was also monitored simultaneously. The oxidisability of LDL isolated from OBJ patients was significantly higher and showed a steep increase in the level of conjugated diene formation without any lag phase. In normal samples the oxidation proceeded slowly with a lag phase. This was also evidenced by the level of formation of hydroperoxides and TBARS. The basal level of LDL α-tocopherol was significantly low in OBJ samples. UDCA was found to delay the oxidation of LDL in a dose dependent manner. The consumption of α-tocopherol was found to be minimum in the presence of UDCA. The results of this investigation show that there is a high susceptibility of LDL to oxidation in OBJ cases and this may be due to low endogenous LDL α-tocopherol content. UDCA minimizes LDL oxidation in dose dependent manner, which is an additional evidence for its antioxidant nature.

1. Introduction

Ursodeoxycholic acid (UDCA), a 7-β-hydroxy epimer of chenodeoxycholic acid is an established drug for the treatment of liver abnormalities associated with obstructive jaundice, chronic cholestasis (Kumar and Tandon 2001), primary biliary cirrhosis (Heathcote 2000) and primary sclerosing cholangitis (Steil 2000). UDCA is hydrophilic in nature and protects directly against the toxicity of hydrophobic endogenous bile salts such as chenodeoxycholic acid and deoxycholic acid which cause hepatobiliary damage due to their detergent action (Benedetti et al. 1997; Mottion et al. 2000). We have already reported the protective effect of UDCA on free radical mediated damage to liver mitochondria and DNA (Geetha and Parameswari 2002; Geetha et al. 2002). The antioxidant nature of the drug has also been reported (Sokol et al. 1993).

The formation, behaviour and scavenging of oxygen free radicals and other oxygen derived species in biological systems has received much attention, with increasing evidence that it is closely connected with a variety of pathological conditions including arteriosclerosis, cancer, arthritis and liver disorders (D'Odorico et al. 1999; Kahler et al. 2001). In these pathological conditions, DNA and lipid damage are of particular importance and have been recognized in a large number of liver diseases (Parman et al. 1999; Miyajima 2000).

LDL oxidation is a crucial step in the development of hyperlipidemia and arteriosclerotic lesions (Hussein et al. 2002). The presence of activated T lymphocytes and macrophages and high expression of human leucocyte antigen (HLA) class 1 and class 11 molecules are indicative of a local immunologic activation in various diseases (Maggi et al. 1993) and this may be closely related to the level of oxidisability of LDL. The oxidized LDL (ox LDL) provokes the formation of anti-ox LDL which are one of the mediator groups of inflammatory reactions. So in this study the level of susceptibility of LDL for oxidation *in vitro* was determined in obstructive jaundice (OBJ) patients and in normal subjects. Since UDCA is a well known drug used for complications arisen due to extra hepatic biliary obstruction, the effect of various concentrations of UDCA on copper induced oxidation of LDL obtained from the blood of jaundiced patients and of normal subjects was also studied.

2. Investigations and results

The incubation of LDL isolated from normal subjects and from OBJ patients with 5 mM $CuSO_4$ resulted in oxidation of LDL with the concomitant formation of conjugated dienes (Fig. 1). In normal subjects, there is a long lag phase after which there was a slow increase in the level of conjugated diene formation. The oxidation profile was characterized by an initial inhibition or lag phase followed by a propagation phase where the rate of conjugated diene formation was increased. In OBJ patients, there was no

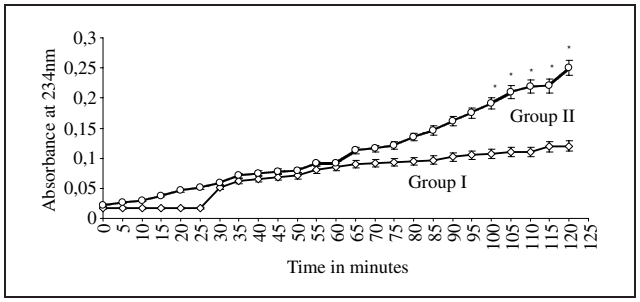

Fig. 1: Time course of conjugated diene formation. An aliquot of 200 µg LDL isolated from control subjects and OBJ patients was incubated with 5 mM $CuSO_4$ in 5 mM Hepes buffer containing 115 mM NaCl, 4 mM $CaCl_2$ and 2 mM $MgCl_2$ in a total volume of 5 ml. Aliquots were withdrawn at different time intervals and conjugated diene was quantified by monitoring the increase in absorption at 234 nm.
Values are expressed as mean ± SD for 20 individual LDL preparations from each group. Statistically significant variations are expressed as $^{*}p < 0.001$ when compared to control samples

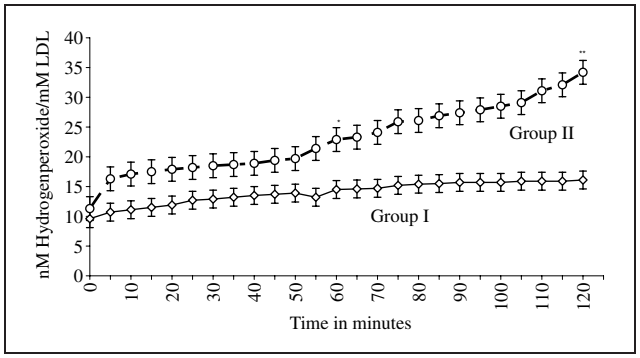

Fig. 2: Time course of hydroperoxide formation. An aliquot of 200 µg LDL isolated from control subjects and OBJ patients was incubated with 5 mM $CuSO_4$ in 5 mM Hepes buffer containing 115 mM NaCl, 4 mM $CaCl_2$ and 2 mM $MgCl_2$ in a total volume of 5 ml. Aliquots were withdrawn at different time intervals and hydroperoxide level was quantified by xylenol orange reagent as mentioned in "Experimental" section.
Values are expressed as mean ± SD for 20 individual LDL preparations from each group. Statistically significant variations are expressed as $^{*}p < 0.01$ and $^{**}p < 0.001$ when compared to control samples

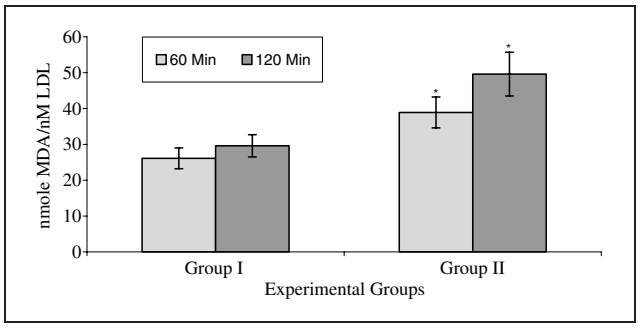

Fig. 3: Time course of TBARS formation. An aliquot of 200 µg LDL isolated from control subjects and OBJ patients was incubated with 5 mM $CuSO_4$ in 5 mM Hepes buffer containing 115 mM NaCl, 4 mM $CaCl_2$ and 2 mM $MgCl_2$ in a total volume of 5 ml. Aliquots were withdrawn at 60 and 120 min and TBARS formation was monitored by malondialdehyde level.
Values are mean ± SD for 20 individual LDL preparations in each group. Statistically significant variations are expressed as $^{*}p < 0.001$ when compared to control samples

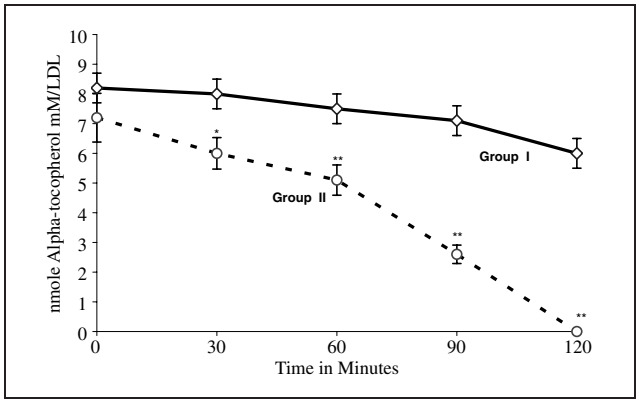

Fig. 4: α-Tocopherol consumption in LDL isolated from control and obstructive jaundice patients. LDL isolated from control subjects and obstructive jaundice patients was subjected to oxidation as described earlier and aliquots were withdrawn at different time intervals and α-tocopherol was assayed as described in section "Experimental"
Values are expressed as mean ± SD for 20 individual LDL preparations in each group. Statistically significant variations are expressed as $^{*}p < 0.01$ and $^{**}p < 0.001$ when compared to control group

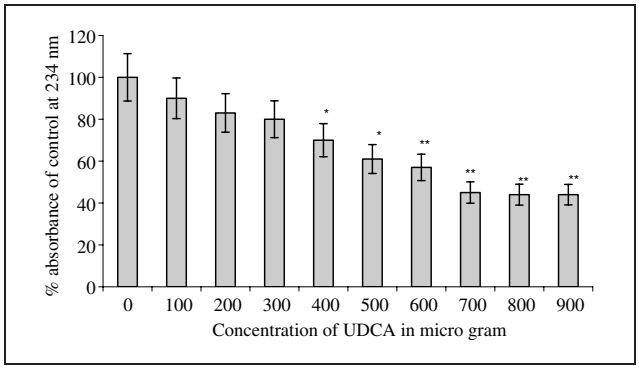

Fig. 5: Effect of UDCA on LDL oxidation. LDL isolated from OBJ patients incubated with UDCA (0–900 µg) for 1 h and oxidation was induced by copper sulphate as described in section "Experimental". LDL oxidation was monitored by conjugated diene formation.
Values are expressed as mean ± SD for 20 individual LDL preparations. Statistically significant variations are expressed as $^{*}p < 0.01$ and $^{**}p < 0.001$ when compared to 0 UDCA concentration

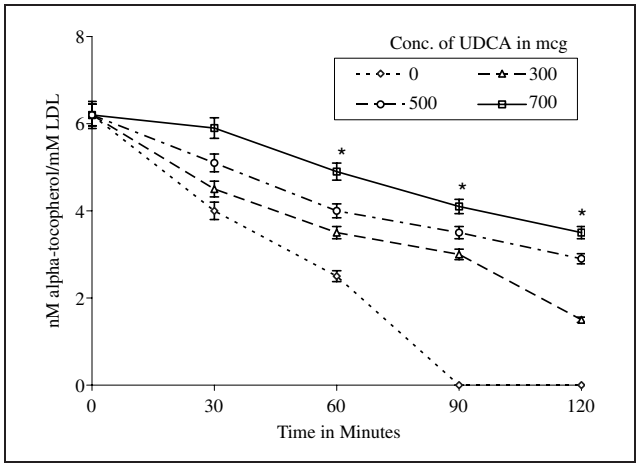

Fig. 6: Effect of UDCA on α-tocopherol consumption in LDL. LDL isolated from OBJ patients incubated with UDCA (0–700 µg) for 1 h and oxidation was induced by copper sulphate as described in section "Experimental". Aliquots were withdrawn at different time intervals and α-tocopherol content was quantified.
Values are expressed as mean ± SD for 20 individual LDL preparations. Statistically significant variations are expressed as $^{*}p < 0.001$ when compared to LDL oxidation without UDCA

lag phase and noted a steep increase in the formation of conjugated diene in propagation phase.

Fig. 2 shows the level of lipid hydroperoxides at different time intervals. There was a gradual increase in the level of lipid hydroperoxides in parallel with the formation of conjugated dienes. Again, there was a significant difference ($P < 0.001$) in the level of lipid hydroperoxides between the LDL isolated from normal and OBJ patients. The oxidation process involved both the lipid and the protein moiety of the lipoprotein particles as known by their increased electrophoretic mobility on nitrocellulose. Similar results were observed (Fig. 3) in the formation of lipid peroxides measured in terms of TBARS.

Fig. 4 presents the level of endogenous LDL α-tocopherol consumption in normal and in obstructive jaundice samples as the oxidation proceeded. There was a significant low basal level of α-tocopherol in LDL of OBJ patients and the rate of consumption was also high when compared to normal subjects ($P < 0.001$).

Fig. 5 presents the effect of various concentrations of UDCA on copper induced LDL oxidation (isolated from group II individuals) measured in terms of conjugated diene formation, and the effect of same on the consumption of endogenous LDL α-tocopherol in group II (Fig. 6). There is a dose dependent increase in the prevention of LDL oxidation which was isolated from OBJ patients. The consumption of α-tocopherol was also minimized in the LDL incubated with different concentrations of UDCA. It also prolonged the duration of the lag phase and decreased the initial rate of endogenous α-tocopherol consumption.

3. Discussion

Several *in vitro* systems have been developed to mimic the reactions occurring *in vivo* and to investigate the susceptibility of isolated LDL for oxidation. Among them, the experimental set up involving the pro-oxidant activity of copper ions has become more and more popular (Bellomo et al. 1995). This system has been successfully employed to investigate the inhibitory effect exerted by a variety of lipid soluble as well as water soluble antioxidants, including α-tocopherol, carotenoids, ascorbic acid and urate on LDL oxidation (Easterbauer 1991).

In vitro, copper binds with LDL which promotes lipid peroxidation (Ziouzenkova et al. 1998). The kinetics of LDL oxidation can be followed by measurement of the increase in the diene absorption and the formation of lipid hydroperoxide and lipid peroxides. The typical time course of the diene formation in LDL can be divided in to three consecutive phases; lag phase, propagation phase and decomposition phase which were well characterized by Esterbaur et al. (1989). LDL isolated from OBJ patients have shown high susceptibility towards oxidation evidenced by the time course of formation of conjugated dienes, lipid hydroperoxides and TBARS.

Increased levels of accelerated generation of reactive oxygen species and toxic degradative products of lipid peroxidation have been reported in the plasma of individuals with hepatobiliary diseases (Tsai et al. 1993). A major target of the various oxidative species occurring in the blood plasma is low density lipoprotein. Oxidation of LDL is a free radical process in which the poly unsaturated fatty acids of LDL are degraded by lipid peroxidation process to a variety of aldehydes (Srivastava et al. 2001).

Our results suggest that LDL in obstructive jaundice patients with hyperlipidemia are susceptible for oxidative damage *in vitro* and the same effects can be expected *in vivo*. The consequences of LDL oxidation have been explained by many workers (Steinbrecher 1988; Karmansky and Yanni 1999). LDL is a heterogeneous particle that contains apolipoprotein B-100, cholesteryl esters, cholesterol, triacyl glycerol and phospholipids. Oxidative modification of the lipid molecules includes degradation of poly unsaturated fatty acid residues and generation of highly reactive aldehydes and other products that can modify other lipids. The cholesterol moiety also undergoes oxidation, and apolipoprotein B undergoes non-enzymatic degradation, as well as derivatization of its lysine and other residues (Hoppe et al. 1997). Several of these modification represents neoantigens that may elicit antibody production (Witztum 1993). Because the LDL oxidation represents the complex and heterogeneous series of reactions, it is likely that many structures are formed that may serve as neoantigens and give rise to many different T and B cell clones reactive against a large variety of epitopes including inflammatory agents causing anti-inflammatory reactions. In cholestasis, anti-inflammatory responses have been reported (Steinberg et al. 1989) and the formation of oxidized LDL may be accounted as one of the causes.

The necessary involvement of lipid hydroperoxides in copper induced LDL oxidation have been further investigated using partially oxidised LDL treated with the combination of ebselen and glutathione, a condition which has been reported previously to reduce lipid hydroperoxides (Steinbrecher 1988). From the results of this investigation, it is inferred that UDCA pre-treatment minimize the oxidative damage of LDL isolated from OBJ patients.

The low level of α-tocopherol would have rendered LDL for Cu^{++} mediated oxidation. The α-tocopherol present in the normal LDL samples might be responsible for the long lag phase and a very slow rise in the absorbance at 233 nm and the formation of lipid hydroperoxides and TBARS. The role of α-tocopherol in preventing LDL oxidation has been well explained by Kontush et al. (1996). Pre-treatment of isolated LDL with UDCA minimized the consumption of α-tocopherol (Fig. 5) and preserve the antioxidant status of LDL.

When the endogenous antioxidants are completely lost and lipid peroxides are formed, it can be predicted that the propagation of lipid peroxidation in the copper model will follow two distinct routes, one involving Cu^{+} as given by the equ.

$$Cu^{+} + LOOH \rightarrow Cu^{++} + OH^{-} + LO^{\bullet}$$

with the concomitant formation of lipid alkoxy radicals which can be quenched by α-tocopherol. The other equation involving

$$Cu^{++} + LOOH \rightarrow Cu^{+} + LOO^{\bullet} + H^{+}$$

where $LOO^{\bullet}$ is the lipid peroxyl free radical. Although these reactions have different rate constants in the presence of endogenous antioxidants like uric acid and α-tocopherol, their operations are strictly depend on (a) the Cu^{++} to Cu^{+} ratio, (b) the rate of reduction of Cu^{++} to Cu^{+} by LDL associated reductants like α-tocopherol and (c) the concentration of LDL derived lipid hydroperoxides (Abuja et al. 1997).

LDL is a well studied target of biological oxidation. Three mechanisms could be considered in the pharmacological antagonism of copper initiated LDL oxidation: scavenging of radical species involve in LDL oxidation, such as peroxyl, alkoxyl or lipid radicals; stabilization of the lipid

moiety of LDL, conceivably via chemical interactions between drug hydrophobic groups and poly unsaturated residues of LDL phospolipids; and metal complexation-inactivation. During the lag phase, antioxidants may protect the poly unsaturated fatty acids of LDL against oxidation by scavenging lipid peroxy radicals. Many studies performed with other water soluble antioxidants such as ascorbic acid (Stait and Leake 1994) trolex and green tea cathechins (Yamanaka et al. 1997) revealed that these compounds may act as antioxidants during copper mediated LDL oxidation. UDCA minimized the oxidative deterioration of LDL presumably by preserving the α-tocopherol content.

The effect of oxidized LDL on the functions of poly morphonuclear lymphocytes and other cells involved in human immune defense mechanism has been well explained (Stemme et al. 1995; Kimura et al. 2001). The ox-LDL stimulate T cell migration but it is immunogenic and induces antibody production. It has been reported that the immune response of jaundiced patients may also be modulated by the anti-inflammatory cytokines and the naturally occurring specific antagonists of TNF and IL-1 (Biffi et al. 1996). The anti-inflammatory mediators seem to be closely related to the immune response of patients with infectious complications. So the formation of oxidized LDL in obstructive jaundiced patients with hyperlipidemia may be considered as one of the factors for the functional impairment of neutrophils in those patients.

One of the mechanisms of action of the drug is closely associated with its antioxidant nature and the hydrophilic property. The hydrophilic and antioxidant property of the drug has been explained by Mitsuyoshi et al. (1999). However, the exact mechanism of action of UDCA on copper induced oxidation of LDL has to be evaluated but we can say that UDCA treatment is advantageous in obstructive jaundice patients with hyperlipidemia which may prevent the risk of LDL oxidation-mediated disturbances.

It could be concluded that the copper- induced oxidisability of low density lipoprotein isolated from obstructive jaundice patients was considerably high when compared to that of normal subjects. The *in vitro* oxidation of low density lipoprotein was delayed and considerably minimized in the presence of UDCA in a dose dependant manner. The consumption of endogenous α-tocopherol was also minimum in the presence of the drug. The pathological consequences of oxidized LDL formation in liver diseases such as transformation of macrophages, mono nuclear cells and polymorph nuclear lymphocytes as neoantigens and immunological reaction against these cells may be prevented when the treatment procedure includes UDCA.

4. Experimental

4.1. Subjects

Patients admitted in the Department of Surgical Gastroenterology and Proctology were diagnosed by abdominal radiography, oral cholecystography and abdominal ultrasonography.

The jaundiced patients with confirmed obstruction due to gall stones either in the gall bladder or in the common bile duct; with cancer in the head of pancreas or carcinoma in the peri ampullary region or; with stricture in the common bile duct were considered in this study (Group II).

The jaundiced patients with cholesterol levels of 300–350 mg/dl were considered in this study. Age and sex matched normal volunteers without any complications including heart, thyroid, kidney and liver related abnormalities were treated as control subjects (Group I). Blood was collected from each patient and plasma was separated.

The protocol was approved by the ethical committee of Stanley Medical College and Hospital, Chennai and the patients gave their informed consent. The patients were of both sexes and in the age group of 30–55 years.

4.2. Methods

4.2.1. Isolation of LDL

Isolation of LDL was performed by the method of Esterbauer et al. (1991). The LDL fraction was isolated from the whole plasma by ultra centrifugation through a potassium bromide discontinuous gradient and collected as the fraction floating at a density of 1.019–1.063 g/ml. Ascorbic acid and uric acid were removed by rapid filtration through disposable desalting columns (Ecano – Pac 10G; Biorad) and LDL was resuspended in PBS (10 mM phosphate, pH 7.2) at the concentrations indicated in the different sets of experiment (assuming that 1 mg of LDL mass = 200 μg of LDL protein/ml). Filtered LDL was immediately employed for oxidation experiments.

4.2.2. UDCA treatment

Aliquots of LDL in PBS were treated with UDCA at different concentrations (0–900 μg) for one hour.

4.2.3. Oxidation of LDL

The method adopted by Bagnati et al. (1999) was followed for the assessment of LDL oxidation. LDL oxidation was performed at 37 °C in disposable polypropylene tubes with continuous gentle shaking. 200 μg of LDL/ml was oxidized by incubating with 5 mM copper II sulphate in 5 mM Hepes buffer containing 115 mM sodium chloride, 4 mM calcium chloride and 2 mM magnesium chloride in a total volume of 5.0 ml for 2 h.

Oxidation of LDL was performed at 37 °C with a diode array spectrophotometer equipped with a 6 position automatic cell changer. Oxidation was inhibited at regular intervals by the addition of butylated hydroxy toluene to a final concentration of 20 μM. Level of oxidation was evaluated by the assay of conjugated dienes (Esterbauer et al. 1989), hydroperoxides (Jiang et al. 1992) and TBARS (Draper and Hadley 1990) at different time intervals. LDL protein was characterized by agarose electrophoresis and quantified by Lowry's method. TBARS values were 19 +/− 3 mM of malondialdehyde equivalents per mg protein for oxidized LDL preparations and less than 2 mM/mg protein for native LDL. LDL was maintained in 20 mM butylated hydroxy toluene to prevent oxidation. All lipoprotein preparations were sterilized by filtration through 0.22 μm Millex Gr Column before use.

4.2.4. Determination of α-tocopherol content

Aliquots of isolated LDL was added to 0.5 ml absolute ethanol and mixed well. The contents were left in the room temperature for 30 min with intermittent shaking. To this mixture 2 ml of hexane was added and vortex-mixed for 2 min. The hexane layer was collected in another test tube and evaporated to dryness. The residue was dissolved in hexane and used for the estimation of α-tocopherol (Taylor et al. 1975).

4.2.5. Statistical evaluation

The values were subjected to analysis of normal tests of significance. RA Fishers Null Hypotheses was adopted to find out the significance of variations between different experimental conditions.

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