ELSEVIER

Biochimica et Biophysica Acta 1254 (1995) 250–256

BBA Biochimica et Biophysica Acta

Oxidation of low-density lipoproteins: effect of antioxidant content, fatty acid composition and intrinsic phospholipase activity on susceptibility to metal ion-induced oxidation

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Received 3 May 1994; revised 8 August 1994

Abstract

The oxidative modification of low-density lipoprotein (LDL) may play an important role in atherogenesis. Our understanding of the mechanism of LDL oxidation and the factors that determine its susceptibility to oxidation is still incomplete. We have isolated LDL from 45 healthy individuals and studied the relationship between LDL fatty acid, vitamin E and β-carotene composition, intrinsic phospholipase A_2-like activity and parameters of LDL oxidation. LDL was exposed to a copper ion-dependent oxidising system and the kinetics of oxidation studied by monitoring formation of fatty acid conjugated dienes. The length of the lag phase of inhibited lipid peroxidation was measured as well as the rate of lipid peroxidation during the propagation phase. There was no significant correlation between LDL antioxidant vitamin or fatty acid composition and lag time to LDL oxidation. Oleic acid was negatively correlated with the rate of LDL oxidation ($r = -0.41$, $P < 0.01$) whilst linoleic acid was significantly correlated with the extent of LDL oxidation measured by the production of total dienes ($r = 0.34$, $P < 0.05$). Interestingly, LDL vitamin E content was positively correlated with both the rate ($r = 0.28$, $P < 0.05$) and extent of LDL oxidation ($r = 0.43$, $P < 0.01$). LDL isolated from this group of subjects showed significant intrinsic phospholipase-like activity. The phospholipase activity, whilst not correlated with lag time, was significantly correlated with both rate ($r = 0.43$, $P < 0.01$) and total diene production ($r = 0.44$, $P < 0.01$) of LDL oxidation. We conclude that antioxidant content, fatty acid composition and intrinsic phospholipase activity have little influence on the lag time of Cu-induced LDL oxidation. These components do however, significantly influence both the rate and extent of LDL oxidation, with increased vitamin E, linoleic acid content and phospholipase activity associated with faster and more extensive oxidation. The possible pro-oxidant effect of vitamin E has interesting implications for the postulated 'protective' effects of vitamin E on atherogenesis.

Keywords: LDL oxidation; Antioxidant; Fatty acid; Phospholipase A_2; (Human)

1. Introduction

There is increasing evidence that the peroxidation of lipoproteins, particularly low-density lipoproteins (LDL), plays an important role in atherogenesis [1,2]. The oxidation of LDL involves peroxidation of fatty acids and covalent modification of apolipoprotein B by fatty acid peroxidation breakdown products. Such modified LDL is readily taken up by macrophages leading to formation of foam cells [3]. Other properties of oxidised LDL also implicate it in atherogenesis. These include stimulation of leukocyte adhesion to vascular endothelium, cytotoxicity, and inhibition of nitric oxide-mediated vasodilation [4–7].

Despite the now substantial evidence linking LDL oxidation to atherosclerosis, our understanding of the mechanisms of LDL oxidation and the factors that determine its susceptibility to oxidative modification is still incomplete.

All four major cell types within atherosclerotic lesions (endothelial cells, smooth muscle cells, macrophages, and lymphocytes) can oxidise LDL [8–10]. The presence of catalytic amounts of transition metal ions such as Cu and Fe may be necessary for these cell based oxidations, and certainly LDL can be oxidised in vitro in cell-free media by the presence of copper ions, for example [8,11]. In fact, the stimulation of isolated LDL peroxidation with Cu ions has become a widely used experimental system to assess LDL oxidation [12]. Such a system may be relevant to events occurring within atherosclerotic lesions, given the demonstrated presence of catalytic copper ions in such

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SSDI 0005-2760(94)00166-9

lesions [13]. The susceptibility of LDL to oxidation in vitro displays a large degree of variation between different subjects [12,14,15]. Interestingly, the levels of predominant lipid-soluble antioxidants such as tocopherol, β-carotene, and ubiquinol do not appear to account for differences in the susceptibility of LDL to oxidation between subjects [16–18]. However, dietary supplementation with vitamin E can increase LDL's resistance to oxidation [19–22]. It is now clear that other factors, apart from antioxidant vitamin content, must determine the susceptibility of LDL to oxidation.

Among these other factors might include the structure of the apoprotein and its degree of glycosylation, the lipid composition of the LDL particle, its fatty acid composition, and particle size and density. The presence of preformed peroxides may also be important given their role in the initiation of lipid peroxidation [12]. In a recent study by Frei and Gaziano, the presence of preformed lipid hydroperoxides in LDL enhanced its oxidisability and accounted for 15% of the variability in susceptibility of LDL to Cu-induced oxidation [23].

The LDL apoprotein B has associated with it a number of enzyme activities, such as phospholipase A_2-like activity [24,25]. This phospholipase activity may play a crucial role in the oxidative modification of LDL inducing generation of lyso phospholipids and release of peroxidised-free fatty acids within the particle [26]. This may have the potential to enhance free radical initiated oxidation and effect the lipoprotein's susceptibility to oxidation. The relationship between LDL's intrinsic phospholipase activity and its susceptibility to oxidation has not previously been investigated.

In order to gain further insights into the factors which might determine the susceptibility of LDL to Cu-induced oxidation, we have isolated LDL from 45 healthy individuals and studied the relationship between fatty acid, vitamin E and β-carotene composition, intrinsic PLA_2-like activity and parameters of LDL oxidation in the form of lag time, rate of oxidation, and formation of conjugated dienes. We conclude from our results that these factors have little influence on LDL lag time to oxidation, but do have a significant impact on the rate and extent of LDL oxidation with increased vitamin E, linoleic acid and PLA_2 activity associated with faster and more extensive oxidation.

2. Materials and methods

2.1. Subjects

45 healthy, non-smoking male volunteers were recruited from the community and all gave their informed consent. Subjects were normotensive with a mean age of 48 years and body mass index 25.8 kg/m^2. None of the subjects were taking supplementary antioxidants or were on drug therapy. After an overnight fast, blood was taken for measurement of routine lipids and for the isolation of LDL.

2.2. LDL isolation

Blood was collected into EDTA (1 mg/ml). LDL was isolated from plasma by density gradient ultracentrifugation [27]. Briefly, plasma density was increased to 1.07 by addition of NaCl and then a 4 step gradient was constructed over the plasma using the following densities: 0.5 ml 1.063 (NaCl), 0.5 ml 1.04 (NaCl), 0.5 ml 1.02 (NaCl) and 0.9 ml H_2O. Samples were ultracentrifuged at 296 000 $\times g$ (average) for 4 h using a Centrikon T-1190 Ultracentrifuge (Kontron Instruments, Milano, Italy). The LDL band was collected by aspiration and passed through a Pharmacia PD10 Sephadex column to remove the excess salt and the majority of the EDTA. The LDL was stored at 4°C in the dark and used for the oxidation studies within 24 h.

2.3. LDL oxidation

The in vitro oxidation procedure used was essentially that described by Esterbauer et al. [28] and has been used by us previously [29]. The isolated LDL was passed through a Pharmacia PD10 Sephadex column to remove the remaining EDTA just prior to the oxidation experiments. The cholesterol concentration of the LDL was measured using a standard enzymatic method (Monotest, Boehringer-Mannheim, Germany) and the LDL diluted with PBS to a standard concentration of 0.3 mmol/l cholesterol. Oxidation was initiated by the addition of freshly prepared aqueous $CuCl_2$ solution (final concentration 2 μM). Oxidation kinetics were determined by monitoring the change in absorbance at 234 nm using a DU650 UV-Vis spectrophotometer (Beckman Instruments, CA, USA) with absorbance readings made every 20 min over 240 min. All experiments were performed at a controlled

Table 1
Details of subjects from which LDL samples were isolated, including plasma lipid profile

	Mean ± S.D.
Age (years)	48 ± 11
BMI (kg/m^2)	25.8 ± 3.0
Cholesterol (mmol/l)	5.42 ± 1.12
LDL chol (mmol/l)	3.59 ± 0.98
HDL chol (mmol/l)	1.24 ± 0.27
Triacylglycerol (mmol/l)	1.22 ± 0.66
Vitamin E (μmol/mmol chol.)	2.32 ± 0.44
β-Carotene (μmol/mmol chol.)	0.09 ± 0.04
Lag time (min)	99.7 ± 18.1
Rate (Δ absorbance/min)	0.013 ± 0.002
Dienes (mmol/mmol chol)	0.18 ± 0.02

Levels of vitamin E and β-carotene in isolated LDL and length of the lag phase, rate of oxidation during the propagation phase and total diene production of LDL exposed to Cu^{2+}. ($n = 45$).

temperature of 30°C. The plot of absorbance against time was divided into three phases, i.e., a lag, a propagation and a decomposition phase, as described by Esterbauer [28]. The lag time was defined as the intercept between the tangent of the absorbance curve during the propogation phase with the baseline and was expressed in minutes. The rate of oxidation was calculated from the slope of the absorbance curve during the propagation phase and was expressed as Δ absorbance units/minute. The total amount of conjugated dienes produced by the oxidation procedure was calculated using maximal absorbance at 234 nm and the Beer-Lambert Law with the molar absorbance of conjugated dienes assumed to be 29500. The results were expressed as total diene (mmol/l) per mmol/l of LDL cholesterol.

2.4. Antioxidant analysis

200 μl of LDL was mixed with 200 μl of cold ethanol, containing tocol (gift from Hoffman-La Roche, Switzerland) as internal standard. The mixture was extracted with hexane (800 μl). The hexane phase was dried under vacuum and reconstituted in ethanol. The extract was analysed by reverse phase HPLC using electrochemical detection as previously described [29]. The mobile phase was methanol/ethanol (50:50, v/v) containing 2.5 g/l sodium perchlorate with a flow rate of 1 ml/min on a 25 cm Nucleosil C18 column (Alltech). Under these conditions, tocol eluted at 4.6 min, tocopherol at 5.6 min and β-carotene at 11.2 min. α-Tocopherol was quantitated against the internal standard after calculation of response factor by injection of standards. β-carotene was quantitated against external standards which were quantified by UV absorption at 452 nm and molar extinction co-efficient of 137000. Values are quoted as mmol of α-tocopherol or β-carotene per mmol of LDL cholesterol for each sample. The co-efficient of variation for the α-tocopherol assay was 0.87% and for β-carotene 4.2%.

2.5. Fatty acid analysis

The fatty acid composition and quantitation of individual LDL samples was determined, following chloroform/methanol (2:1, v/v) extraction, by gas chromatography of the methyl ester derivatives as previously described [30]. Samples were run using a BPX 70 25 m $\times$ 0.32 mm capillary column (SGE), temperature programme from 150°C to 210°C at 4° per min. Heptadecanoic acid was used as an internal standard and fatty acid quantitated per mg of LDL protein.

2.6. Phospholipase activity

We used a modification of the method of Parthasarathy et al. [24] to measure PLA_2 like activity in LDL. Briefly, to 200 μg of LDL protein, were added 200 μl of 0.2 M Tris buffer (pH 7.2) containing 1 mg of Na deoxycholate and 200 μl of $CaCl_2$ to a final concentration of 1.5 μM. This was made up to a final volume of 800 μl with 0.2 M Tris buffer (pH 7.2) and preincubated at 37°C for 15 min. 40 nmol of 1-palmitoyl-2-[1-^{14}C]linoleoyl-*sn*-glycero-3-phosphocholine oxidised with soybean lipoxygenase [26] was added to the reaction mixture and incubated for 2 h at 37°C. After incubation, samples were extracted by the method of Bligh and Dyer using 1 ml of 1 M HCL for acidification. The chloroform phase was separated and dried under N_2. The fatty acid products were separated by TLC using the solvent; chloroform/methanol/acetic acid/water (90:10:0.5:0.5, v/v). Oxidised linoleic acid and linoleic acid were run as standard carriers. Identification of the fatty acid products was by brief exposure to iodine vapours. The bands corresponding to linoleic acid and oxidised linoleic acid were scraped into plastic beta vials to which 500 μl of methanol was added to elute the fatty acids off the silica. Five mls of scintillant (Optiphase) was added and the samples counted for radioactivity. Apparent enzyme activites are expressed in terms of nmol of labelled free fatty acid released per mg of protein per 2 h. LDL protein was measured by the Lowry method.

2.7. Statistics

All variables were confirmed normal in distribution in this study group. Relationships between variables were examined using linear regression analysis using Statistical Package for Social Sciences (SPSS).

3. Results

Details of the subjects participating in this study including lipid levels, LDL antioxidant status and indices of LDL oxidation are given in Table 1. Subjects were normotensive and had blood lipids in the normal range. The vitamin E and β-carotene content of LDL was determined in samples frozen at -80°C for less than 12 weeks. Antioxidant vitamin levels in LDL were similar to those previously reported by us [29] and others [16]. The values reported for lag time are within the range of that obtained by a number of other workers (reviewed by Esterbauer [12]). No doubt some of the variation between laboratories in estimates of lag time determination is due to differences in oxidation conditions such as concentration of LDL, Cu^{2+}, and the temperature at which the oxidation is conducted. We have carefully standardised our conditions which are similar to those recommended by Kleinveld et al. [31].

The fatty acid composition and quantitation for the LDL samples is given in Table 2, with results expressed as % composition and mg of fatty acid per mg of LDL protein. LDL is particularly rich in linoleic acid which is no doubt the major substrate for peroxidation. The percent composi-

Table 2
Fatty acid composition of LDL expressed as absolute ammount and as % composition

	μg/mg protein	%
Palmitic acid (16:0)	677 ± 260	26.7 ± 3.7
Stearic acid (18:0)	367 ± 228	14.5 ± 5.6
Oleic acid (18:1)	401 ± 95	15.8 ± 3.2
Linoleic acid (18:2)	889 ± 204	35.0 ± 6.1
Arachidonic acid (20:4)	170 ± 43	6.7 ± 1.6
Docosahexaenoic acid (22:6)	33 ± 16	1.3 ± 0.6

Heptadecanoic acid was used as internal standard and results are expressed as μg/mg LDL protein ± S.D. ($n = 45$).

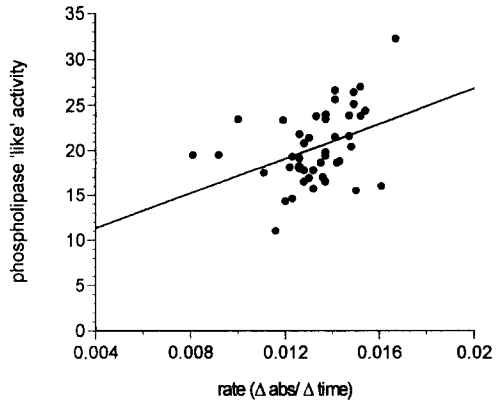

Fig. 1. Scatterplot showing the relationship between phospholipase-like activity (nmol/mg protein per 2 h) and rate of LDL oxidation (Δ abs/Δ time). $r = 0.43$, $P = 0.008$.

tion of fatty acids in LDL are similar to those reported by other workers [15,32].

Vitamin E and β-carotene levels in LDL were not significantly correlated with each other and there was also no significant correlation between these antioxidant vitamins and LDL oxidation lag time. The LDL vitamin E level was also calculated in terms of LDL protein and linoleic acid content (8.58 ± 3.8 nmol/mg protein and 9.76 ± 3.6 nmol/mg linoleic acid) and vitamin E – lag time relationships using these different reference bases studied. There was however no significant correlation between indexes of LDL oxidation and vitamin E expressed in terms of protein or linoleic acid. Vitamin E content of LDL (per mmol cholesterol) was, however, positively correlated with total diene production ($r = 0.43$, $P = 0.002$), and rate of oxidation ($r = 0.28$, $P < 0.05$) indicating that, while tocopherol is not affecting the susceptibility of LDL oxidation, it may promote the rate and extent of fatty acid oxidation in this model. In some situations tocopherol has been shown to act as a pro-oxidant in lipoprotein oxidations in vitro [33,34]. There were no significant correlations between vitamin E, β-carotene, or lag time and rate with either body weight, blood pressure, or HDL levels of subjects from which the LDL was obtained.

There was no significant correlation between levels of any of the fatty acids and lag time to propagation of LDL oxidation. Oleic acid (18:1($n - 9$)) was negatively correlated with the rate of LDL oxidation ($r = -0.41$, $P = 0.005$) while linoleic acid (18:2($n - 6$)) was not correlated with oxidation rate (Table 3). Linoleic acid was significantly correlated to total diene production ($r = 0.34$, $P = 0.02$), as would be expected since it is a major substrate for fatty acid peroxidation and diene production.

Phospholipase A_2-like activity in LDL was measured using a lipoxygenase-oxidised linoleoyl phosphatidylcholine substrate [26]. Significant phospholipase activity was observed (Table 4) similar to that previously reported by Parthasarathy [26]. The activity was inhibited by 30% with 500 μM *p*-bromophenacyl bromide (an inhibitor of PLA_2 activity), indicating that this activity may involve other enzyme activities such as PAF acetyl hydrolase, for example [35]. For convenience, we have referred to the activity measured as 'PLA_2-like' activity. The PLA_2-like activity was negatively correlated to the palmitic and stearic acid components of LDL ($r = -0.3$ and -0.36, respectively, Table 4). There was no correlation between PLA_2-like activity and lag time to oxidation but the rate of oxidation was significantly correlated with this activity

Table 3
Correlation coefficients (*r*-values) between three indexes of oxidisability and LDL composition

	Vitamin E	β-Carotene	Oleic acid	Linoleic acid	Arachidonic acid
Lag time	−0.05	0.09	0.04	0.11	0.15
Rate	0.28 *	0.08	−0.41 **	0.12	0.08
Dienes	0.43 **	−0.05	0.11	0.34 *	0.16

Significance levels are: * $P < 0.05$, ** $P < 0.01$.

Table 4
Phospholipase activity determined in 45 individual LDL samples, expressed as nmol of labelled fatty acid released per mg of LDL protein in 2 h

	Lag time	Rate	Dienes	Palmitic acid	Stearic acid	Linoleic acid	Arachidonic acid
PLA_2 activity (20.3 ± 4.1)	−0.11	0.43 **	0.44 **	−0.30 *	−0.36 *	0.10	−0.10

Correlation coefficients (*r* values) for phospholipase A_2-like activity and LDL oxidisability and fatty acids.
* $P < 0.05$, ** $P < 0.01$.

($r = 0.43$, $P = 0.008$, Fig. 1), i.e., the greater the phospholipase-like activity associated with the LDL, the faster it oxidises once it enters the uninhibited phase of lipid peroxidation. Total diene production was also significantly correlated with PLA_2-like activity ($r = 0.44$, Table 4).

4. Discussion

The aim of this study was to examine some components of human LDL that may contribute to its susceptibility to, and rate of, oxidative modification. The method used to examine LDL oxidation was the model of Cu-induced oxidation of LDL (adjusted to a standard cholesterol concentration) and monitoring the formation of fatty acid conjugated dienes. This widely used experimental system may be relevant to events occurring within the atherosclerotic lesion given that 'catalytic' copper ions may exist in such lesions [13]. However, results from such model systems must be interpreted with caution since the actual mechanism of LDL oxidation in vivo is still poorly understood, and the factors which determine the susceptibility of LDL to oxidation are also incompletely understood [12,36].

Vitamin E is quantitatively perhaps the most important lipophilic antioxidant in LDL. When vitamin E is taken by mouth, its concentration in LDL can be increased several fold, and this vitamin enriched LDL is less susceptible to oxidation [19–22]. Moreover, there is epidemiological evidence that high vitamin E and β-carotene intake is associated with a low rate of coronary heart disease [37,38]. However, in native unsupplemented LDL, the in vitro susceptibility of LDL to oxidation in this and other studies does not appear to be related to either vitamin E or β-carotene content [16–18]. In this study we have also failed to see any significant correlation between the vitamin E or β-carotene content of LDL and its lag time to oxidation. While many studies report LDL antioxidant vitamin content per mg of protein, we report our results based on the cholesterol (lipid) content of the LDL. In a recent study by Frei and Gaziano, [23] based on 61 LDL samples isolated from 16 individual subjects, they suggest that it is more appropriate to quantitate antioxidant vitamin levels relative to LDL cholesterol. This is because the lipophilic antioxidants can be presumed to primarily protect the lipids in LDL against oxidative attack, and secondly, measurement of lipid peroxidation (which is an important early step in LDL oxidation) is used as an indicator of LDL's susceptibility to oxidation. In the study by Frei and Gaziano [23], while there was no association between LDL susceptibility and vitamin E expressed per mg protein, they did find a significant correlation between lag time to Cu-induced oxidation and vitamin E expressed per mole of cholesterol ($r = 0.42$). In our study, we found no significant correlation between LDL vitamin E expressed per mole of cholesterol and lag time. One reason for this difference may be that we adjust our LDL samples to a standard cholesterol concentration prior to oxidation, while Frei and Gaziano's results were obtained from oxidations carried out at a standard protein concentration. We have also determined vitamin E expressed relative to LDL protein and linoleic acid content, in both cases there being no significant correlation between vitamin E and any index of LDL oxidation.

The rate of LDL oxidation was positively correlated with the vitamin E content of LDL. While this may appear to be incongruent with the role of vitamin E as an antioxidant, recent studies have clearly shown that in several in vitro model systems vitamin E can act as a pro-oxidant [33,34]. Once vitamin E is oxidised, the tocopherol radical can act as a chain-transfer agent rather than as a radical trap, particularly in the absence of other antioxidants such as ascorbate or ubiquinol, which may act to regenerate vitamin E from its radical [39]. LDL vitamin E was also positively correlated with total diene production providing further evidence of it acting in a pro-oxidant capacity in this system. If similar effects operate in vivo in the arterial wall this might mitigate against any protective effect of vitamin E against atherosclerosis arising from its antioxidant potential for LDL in circulating plasma, although it should be acknowledged that any extrapolation of in vitro findings to the in vivo situation must be very tentative at this stage.

Since the polyunsaturated fatty acyl side chains of cholesterol esters and phospholipids in LDL are the main substrates for lipid peroxidation, one might expect that the levels of these fatty acids will be associated with the oxidative susceptibility of the LDL. In several dietary supplementation studies in both rabbits and man, increasing the monounsaturated oleic acid content of LDL and reducing the linoleic acid content has reduced the susceptibility of the LDL to oxidation [15,40,41]. However, there have been limited studies which have looked at LDL fatty acid composition in the baseline state in humans. In a recent report by Kleinveld et al. [32], the ratio of oleic acid to linoleic acid content of LDL was positively correlated with the lag time and inversely correlated with oxidation rate in vitamin E deficient subjects. Thus, LDL rich in oleic acid and poor in linoleic acid was less easily oxidised, a result in keeping with observations in dietary supplementation studies. Our results based on a relatively large number of healthy male subjects show no correlation between either oleic, linoleic, or arachidonic acid levels in LDL and lag time to oxidation. Oleic acid levels in LDL were, however, inversely correlated to the rate of LDL oxidation, indicating that LDL rich in oleic acid is less rapidly oxidised. Linoleic acid content of LDL was significantly correlated to diene production, as would be expected, since linoleic acid is the major substrate for oxidation. Our results suggest that while fatty acid content of LDL may have a major influence on the rate of oxidation and diene production, it does not have a major effect on LDL resistance to oxidation. In a recent study by Thomas

et al. [42] in nonhuman primates fed diets enriched in different types of fatty acids, it has been shown that the rate of LDL oxidation is dependent on the polyunsaturated fatty acid concentration of the LDL.

Another important feature of the present study has been the investigation of an association between LDL PLA_2-like activity and its oxidation. LDL has been shown to have an intrinsic PLA_2 activity associated with apoprotein B-100 [24]. Moreover, it has been suggested that this PLA_2 activity may play an essential role, particularly in cell mediated LDL oxidative modification [26]. Our results indicate that intrinsic PLA_2 activity is significantly and positively correlated with the rate of induced LDL oxidation, but is not significantly related to oxidative resistance (lag time). The presence of an active phospholipase in LDL could release peroxidised-free fatty acids and thus further the free radical initiated peroxidation by favouring propagation reactions within the LDL particle, or by the escape of peroxidised fatty acids which may interact with other LDL particles.

A number of other factors must also be considered as playing a role in the susceptibility of LDL to oxidation. These include the size and density of the LDL particle, with the general observation that the small dense LDL subfractions are more susceptible to oxidation [43]. Frei and Gaziano, on the other hand, found that LDL's with an increased lipid-to-protein ratio (i.e., less dense) were more susceptible to oxidation [23]. Another factor which these same workers investigated was the level of preformed lipid peroxides in the LDL which was found to explain about 15% of the LDL's susceptibility to oxidation. It is likely, then, that the overall susceptibility of LDL to oxidation is determined by a number of both compositional and structural factors. Given the widespread use of the in vitro Cu-induced LDL oxidation model to study LDL oxidative susceptibility in a number of clinical studies, it is critical that we develop a clearer understanding of this process and how it relates to the possible mechanism of LDL oxidation in vivo.

In summary, the findings reported here show that antioxidant content, fatty acid composition, and intrinsic PLA_2 activity have little influence on the susceptibility (lag time) of LDL to oxidation. These components do however, significantly influence both the rate and extent of LDL oxidation, with increased vitamin E, linoleic acid content, and PLA_2 activity associated with both faster and more extensive LDL oxidation.

Acknowledgements

We would like to acknowledge the financial support of the National Health and Medical Research Council of Australia and the Merck Sharp and Dohme Research Foundation.

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