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Seasonal Variation in Copper-mediated Low-density Lipoprotein Oxidation *In Vitro* is Related to Varying Plasma Concentration of Oxidised Lipids in Summer and Winter

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The seasonal variation of $CuCl_2$-mediated low density lipoprotein (LDL) oxidation (10 μM Cu^{2+}, lag phase, rate of oxidation and maximum absorbance at 234 nm) were measured in 43 men and women on 4–6 occasions (mean 5.7 ± 0.5) over a 12-month period.

The lag phase averaged 52.7 ± 0.6 min and did not differ by gender. Lag phase and rate of the rapid propagation phase of LDL oxidation showed a sinusoidal pattern over the year (increased and reduced oxidative susceptibility during January and June–July, respectively; both $p < 0.001$). Changes in plasma α-tocopherol, ascorbic acid, lycopene or β-carotene concentrations did not explain seasonal differences in oxidative susceptibility of LDL *in vitro*. Nor did plasma lipid content of linoleic acid, the main substrate of lipid peroxidation, vary. However, the amount of hydroperoxy- plus hydroxy-fatty acids in plasma lipids varied according to season ($p < 0.024$) and was related to the lag phase ($r = -0.26$, $p < 0.001$). Seasonal variation in oxidative susceptibility was not significant after adjusting for hydroperoxy- plus hydroxy-fatty acids ($p = 0.506$). Isolated LDL is more vulnerable to Cu^{2+}-induced lipid peroxidation during the winter and this may be due to the higher amount of oxidised lipids during that period.

Keywords: Oxidative susceptibility; Hydroperoxy fatty acids; LDL; Seasonal variation; Antioxidants; Lipid peroxidation

INTRODUCTION

The hypothesis that the modification of low-density lipoprotein (LDL) in the subendothelial space of coronary arteries is crucial before this particle becomes truly atherogenic and leads to coronary heart disease receives much support (For recent review, see Ref. [1]). For instance, antibodies raised against oxidised LDL stain atherosclerotic lesions in humans[2] and LDL isolated from atherosclerotic lesions has an electrophoretic mobility characteristic of oxidised LDL.[3] The observation that a low intake of antioxidant vitamin E in populations is associated with an increased risk of coronary heart disease during follow-up also seems to support this hypothesis.[4–7]

The fate of LDL in arterial wall is not easy to determine. It is much easier to monitor Cu^{2+}- or free radical-mediated lipid peroxidation of LDL *in vitro* by the formation of conjugated dienes at the specific wavelength of 234 nm.[8] This method has been extensively used to examine the effect of antioxidant vitamin supplements, dietary fatty acids or drugs, that might alter the balance on the oxidative susceptibility of LDL in humans.[9–13] It has also been applied to examine the role of oxidative modification of LDL in coronary disease related conditions such as endothelial dysfunction, atherosclerosis and diabetes.[14–18] Small dense LDL has been associated with an increased risk of coronary disease. Cu^{2+}-mediated formation of cholesterylester hydroperoxides is increased in the small dense LDL when compared to the other fractions of this cholesterol-rich lipoprotein class.[19]

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An increased susceptibility to oxidation was related to the severity of atherosclerosis in survivors of coronary disease,[20] but the confounding influence of α-tocopherol and linoleic acid content was not considered. Vitamin E supplements inhibit copper-induced oxidation of LDL *in vitro*, although the response to vitamin E in terms of change in lag phase until the rapid oxidation varies considerably between individuals.[9] Seasonal variations in plasma concentrations of vitamin C (more so than of vitamin E) have been reported in several countries.[21–23] There was no seasonal effect on Cu^{2+}-mediated lipid peroxidation of LDL isolated on 3–4 occasions (February, May, September and December) in 10 Dutch volunteers.[22]

In this paper, we report the influence of season on the tendency to lipid peroxidation of isolated LDL in relation to variations in plasma linoleic acid, antioxidant vitamins and oxidised fatty acid levels.

METHODS

Population Sample and Procedures

Volunteers (22 husbands and wives, or partners living together), aged 26–75, were recruited from the population of Edinburgh by advertising in a local newspaper. For this part of the study all subjects were asked to attend a clinic on six occasions approximately 2 months apart over a 12-month period. All subjects were in the fasting state from 9:00 p.m. of the previous evening. One male subject attended only on two occasions, so the LDL-oxidation results relate to 43 men and women. Some of the demographic data are summarised in Table I. The Lothian Research Ethics Committee approved the study.

Blood samples, for the isolation of low density lipoprotein (LDL) particles, and the determination of plasma lipids,[24] their fatty acid composition,[25] and fat soluble vitamins,[26] were collected into EDTA tubes (EDTA K 26.358 PS, Sarstedt, Leicester, UK). Plasma was prepared by centrifugation at 1500*g* for 10 min in a refrigerated centrifuge (Centra 7R, Life Sciences Ltd, Basingstoke, UK). Another blood sample was transferred into a heparinised Eppendorf tube for the determination of plasma vitamin C. The tube was immediately centrifuged in a Hettich microcentrifuge (Type 2020, AR Horwell Ltd, London, UK) for 30 s at 15,000*g*. Plasma (0.5 ml) transferred to another tube was stabilised with 0.5 ml freshly prepared metaphosphoric acid (5%) and frozen on solid CO_2 without delay. Samples were stored at −70°C until analysed using an enzymatic fluorimetric method with the aid of ascorbate oxidase and *O*-phenylenediamine as the coupling agent.[27]

Isolation of LDL

LDL was isolated from plasma using a rapid ultracentrifugal micro method,[28] with the aid of a Beckman TL100 bench top ultracentrifuge and a TLV100 rotor. Briefly, the density of plasma (0.57 ml) was adjusted to 1.21 g/ml by the addition of 186 ± 3 mg KBr. Saline (1.35 ml) with a density of 1.006 g/ml containing 0.01% Na_2-EDTA was then layered above the plasma. The sealed tube was centrifuged at 100,000 rpm for 30 min (7°C) and the clearly visible, orange LDL band was removed. The LDL fraction, after addition of KBr (as above), was recentrifuged to remove any traces of albumin. The purified LDL band was isolated and dialysed overnight against phosphate buffered saline containing Chelex 100 resin (0.2 g/l, Biorad Laboratories, Hemel Hempstead, UK) using washed Spectra/Por tubing molecular weight cut-off 500,000 (Medicell, London, UK). The temperature was 4°C. LDL protein concentration was determined using the biuret reaction according to Lowry on a Cobas Bio centrifugal analyser (Roche Diagnostics, Welwyn Garden City, UK). LDL cholesterol was analysed using a commercial kit (CHOD-PAP No 236691, Boehringer Mannheim, Mannheim, Germany) with the aid of the centrifugal analyser.[24]

Lag Phase Determination

The determination of the onset of the rapid phase of copper induced lipid peroxidation (lag phase) was essential according to Esterbauer *et al*.[8] Four simultaneous determinations were made using a Pye Unicam SP1800 spectrophotometer, equipped

TABLE I Basic characteristics of the male and female volunteers

	Men ($n = 22$)	Women ($n = 22$)	P value
Age (yr)	44.0 ± 2.6	42.9 ± 2.5	0.06
% Current smoker	9	0	
Cholesterol (mM)	5.7 ± 0.09	5.7 ± 0.11	NS
HDL cholesterol (mM)	1.25 ± 0.02	1.59 ± 0.03	<0.001
TG (mM)	1.4 ± 0.06	1.1 ± 0.04	<0.001
BMI (kg/m^2)	23.8 ± 0.7	24.6 ± 1.1	NS
Adipose linoleate (% FA)*	14.1 ± 0.7	13.5 ± 1.4	NS

* Percent of fatty acids of adipose triglycerides.

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with a sample changer and thermostat set at 37°C. The oxidation of LDL (62.5 ng in 1.0 ml phosphate buffered saline) was initiated by the addition of 25 μl freshly diluted $CuCl_2$ (final concentration 10 μmol/l). The formation of conjugated dienes was followed at 234 nm in quartz microcuvettes for at least 2 h. Results were expressed as lag phase (min), rate of oxidation during the rapid oxidation period (OD/min) and maximum change in absorbance (ΔOD_{max}).

Determination of Oxidised Lipids

Oxidised lipids in plasma, collected during visit 2–6, were determined by a capillary gas chromatographic–mass spectrometric method.[29] Briefly, plasma lipids were extracted into 10 ml dichloromethane:methanol 2:1 containing 0.05% (BHT). Hydroxy-nonadecanoic acid (8 μg) and hydroxy-heptadecanoic acid (2 μg) were added as internal standards. The solvents were evaporated under argon and the lipid extract immediately hydrogenated in a reaction catalysed by platinum. After saponification, fatty acids are methylated using diazomethane and the monohydroxy fatty acid methyl esters were separated from saturated and polyhydroxy fatty acyl esters using 500 mg silica SPE columns (Jones Chromatography, Hengoed, UK). The hydroxy-group was converted into a methoxy-group using 0.5% tetramethylammonium hydroxide in methanol: dichloromethane. This reagent methylates alcohols by pyrolysis in hot injection ports (280°C) quantitatively. The methoxy methyl esters were chromatographically resolved using a 25 m × 0.25 mm i.d. CP Sil 19 fused capillary column (Chrompack, Middelburg, the Netherlands). The column effluent was subjected to electron impact mass spectrometry (70 eV) at a vacuum of 170 mTorr. Each methoxy methyl ester was quantified by integration and summation of the two main ions formed by fragmentation of the molecule at either side of the methoxy-group.

Because hydroperoxy fatty acids are converted to hydroxy fatty acids in our method using Pt/H_2, we cannot distinguish between hydroperoxy and hydroxy fatty acids, thus the sum of these are presented. The principal oxidation products of plasma lipids are derived from 9,12-*cis*, *cis*-linoleic acid and the results presented here is the sum of 9-, 10-, 11-, 12- and 13-methoxy stearyl methyl esters. The ratio of the two internal standards eluting before and after the various methoxy stearyl methyl esters is used to monitor the stability of the mass spectrometer.

Statistical Analysis

Results are presented as mean ± standard error of the mean. Statistical analyses were carried out using MINITAB (version 9.2, CLE.COM, Birmingham, UK). Statistical analyses between males and females were carried out using the Student *t*-test and chi square test for continuous and categorical data, respectively.

Seasonal variations were analysed using one-way analysis of variance of continuous variables against month of the year. Significant results were further analysed by regression analysis of the lag phase, oxidation rate or peak absorbance versus the cosine value of the day of the year, expressed as a radian. A p value of less than 0.05 was taken to indicate significance.

RESULTS

The time to the start of the rapid phase of lipid peroxidation (lag phase) measured at 234 nm was some 52.7 ± 0.6 min, and did not differ between the two sexes. Nor did the rate of lipid peroxidation or the maximum of absorbance differ between men and women. Therefore male and female results were combined for further analysis. The lag phase showed a seasonal variation and the susceptibility to lipid

TABLE II Selected plasma antioxidant vitamins, oxidised lipids and LDL oxidation in male and female volunteers and seasonal effects

				Seasonal effect	
	Men	Women	*p* value	Max/Min	*p* value
Ascorbic acid	60 ± 2	72 ± 2	< 0.001	–	0.883
α-Tocopherol	30.4 ± 0.6	30.1 ± 0.7	NS	–	0.248
Lycopene	0.91 ± 0.04	0.79 ± 0.04	0.04	–	0.675
β-Carotene	0.29 ± 0.02	0.44 ± 0.02	< 0.001	–	0.681
Plasma lipid fatty acids					
Oxidised fatty acids (μM)	1.11 ± 0.10	0.98 ± 0.07	NS	Jan/June	< 0.024
Linoleic acid (% FA)*	32.5 ± 0.4	33.7 ± 0.4	0.036	–	0.540
Cu-induced oxidation†					
Lag phase (min)	53.1 ± 0.9	52.3 ± 0.8	NS	June/Jan	< 0.001
Rate (OD/min)	0.034 ± 0.001	0.035 ± 0.001	NS	Jan/June	< 0.001
Peak absorbance (OD)	1.09 ± 0.01	1.10 ± 0.01	NS	–	0.455

*Percentage of fatty acids in plasma lipids. †LDL oxidation *in vitro*.

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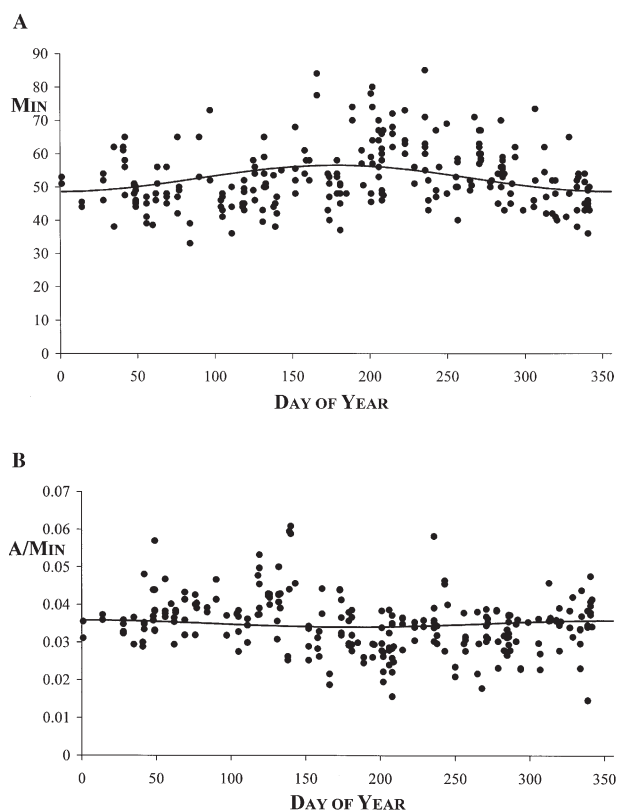

FIGURE 1 Seasonal variation in the duration of the lag phase until the rapid oxidation of isolated LDL (panel A) and rate of LDL oxidation (panel B). LDL was isolated every 2 months on six occasions from 43 apparently healthy subjects. The oxidation of LDL (62.5 ng) was induced by addition $CuCl_2$ (10 μmol/l) at 37°C and monitored at 234 nm for up to 2 h. The trend line was calculated by regression analysis of the data against the cosine of the day. LDL lag phase is longer (more resistant to oxidation) in June–July ($p < 0.001$). The seasonal variation in the rate of oxidation (reduced in the summer) is less pronounced (Panel B, $p < 0.001$). The seasonal variation is no longer observed after adjustment for the concentration of hydroperoxy- plus hydroxy-fatty acids in plasma lipids.

peroxidation increased during the winter months (Table II). The same was observed if the lag phase was expressed as a percentage of the mean lag phase of that individual (ANOVA, F value 10.84, $p < 0.001$). The variation in susceptibility to lipid peroxidation was sinusoidal and related to the cosine of the day of the year (Fig. 1A).

The rate of lipid peroxidation showed a similar seasonal pattern, but the maximum absorbance at 234 nm did not (Table II). In an attempt to explain this seasonal effect we examined antioxidant vitamins and linoleic acid concentrations in plasma. Seasonal effects were not observed (Table II). The presence of hydroperoxy- plus hydroxy-fatty acids (measured on 71% of occasions) also showed a seasonal variation (Table II). The inverse relationship between lag phase and oxidised fatty acids was significant (Table III). The seasonal variation in the lag phase and in the rate of LDL oxidation was no longer observed after adjustment for changing patterns in

TABLE III Correlation coefficients for the relationships between the lag phase and rate of LDL oxidation and plasma oxidised lipids in human volunteers

	LDL oxidation		
	Lag phase	Rate	Oxidised lipids
Lag phase	–	−0.38***	−0.26***
Rate of oxidation		–	NSξ

ξ $p = 0.127$; *** $p < 0.001$.

hydroxy- plus hydroperoxy-fatty acids ($p = 0.506$ and $p = 0.127$, respectively).

DISCUSSION

Our study shows that the susceptibility of isolated LDL to lipid peroxidation is increased during the winter months. A similar 4 min reduction in lag phase from September to December was not significant in the small Dutch study.[22] The susceptibility to oxidation has been related to plasma (or LDL) vitamin E following supplementation.[9,10] In our study plasma antioxidant vitamin levels did not vary significantly in this group of volunteers over the 12-month period. In a previous study of a random population sample of 394 middle-aged Edinburgh men, plasma vitamin E levels were 15% higher not lower in the winter months.[23] The largest variation observed in that study was for plasma vitamin C (48% lower in early spring). Our subjects did provide us with dietary information using 7-day weighed records at the time of each measurement. We will report elsewhere that a single 7-day weighed record is inadequate to document an individuals antioxidant intake.[30] Our impression is that the dietary patterns remained relatively constant over the 12 month period in this group, consisting mostly of social class I and II. Whether the lack of smokers in this study might also have contributed to our lack of seasonal variation in plasma vitamins, we do not know. Smoking is known to interfere with vitamin C absorption[31] and might therefore accentuate seasonal differences in vitamin C intake, which is generally lower in social class III, IV and V than in I and II.[32]

The other explanation for the variation in susceptibility might have been variations in the main substrate of LDL oxidation, linoleic acid.[11,22] The relative or absolute amount of this polyunsaturated fatty acid in plasma did not vary. It could have been argued that we did not measure the fatty acid composition of LDL directly. However, most of the linoleic acid in plasma is derived from cholesterol esters originating from LDL and the fatty acid composition of LDL did not change according to season in the Dutch study either.[22] So the two most

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obvious possibilities fail to explain the increased susceptibility of LDL to lipid peroxidation during the winter months. The fact that peak absorbance at 234 nm, which reflects the amount of linoleic acid in LDL[9,10,16] did not vary throughout the year, supports the view that the seasonal variation in lag phase or oxidation rate is real.

Plasma levels of hydroperoxy- plus hydroxy-fatty acids varied throughout the year and were related to the susceptibility of LDL oxidation in our study. After adjustment for this, the seasonal variation in LDL oxidation disappeared. Thus hydroperoxy fatty acids might have primed LDL for accelerated copper-induced lipid peroxidation.[1] However, the peroxides may have been lost during the isolation and dialysis procedures. Thus more direct evidence is necessary to conclude that the increased level of hydroperoxy fatty acids in plasma lipids caused the seasonal instability of LDL. For instance it would have helped if we could have measured this reactive species in the isolated LDL fraction itself.

It is also possible, although less likely, that the level of hydroperoxy fatty acids could have reflected that of other reactive species that were responsible for the increased tendency to lipid peroxidation. These include epoxides, keto-cholesterol or prostaglandin like substances (isoprostanes). None of those were measured, but with the exception of fatty acyl epoxides, these compounds are end-products, not active players in lipid peroxidation. Transition metals encourage the initiation of lipid peroxidation by the formation of two free radicals from hydroperoxy fatty acids via the Fenton reaction. Thus there is also a good mechanism to explain our results.

There was no difference between men and women in the susceptibility of LDL to lipid peroxidation (and uptake by macrophages) in an earlier study.[33] Our results confirm this. It is important to point out that the men and women in our study were partners and had similar diets and shared most of the foods in the household. The seasonal variation in the susceptibility of LDL to lipid peroxidation and in the plasma levels of hydroperoxy- plus hydroxy-fatty acids is real, but the magnitude is relatively small. Our self-selected population is not representative of the Edinburgh community at large. Our study does not allow us to predict what seasonal effects would have been observed in a random population sample or in groups at high risk of coronary heart disease such as men from the lower socioeconomic class.

Our gas chromatographic–mass spectrometric method may be considered a highly specific way to determine hydroperoxy fatty acids, but this is strictly not so. The catalytic hydrogenation converts all hydroperoxy fatty acids to hydroxy fatty acids. If there were hydroxy fatty acids in plasma lipids then they would have been included in the measurement also. As yet we do not know the relative amounts of the hydroperoxy- and hydroxy-fatty acids in human plasma. Nevertheless, it appears that the presence of products originating from lipid peroxidation in the plasma of a subject reflects the fact that particles may be made vulnerable to copper-induced oxidation. It is worth mentioning that the concentration of thiobarbituric acid reactive substances (TBARS) did not vary throughout the year (not shown). TBARS (which are formed from lipid peroxidation) were measured by a fluorimetric method, that is increasingly being discredited as a measure of circulating plasma malondialdehyde levels.

Our study was commenced out of our interest in the role of oxidised LDL in the genesis of atherosclerosis and coronary heart disease. One of the main difficulties in testing this hypothesis remains the lack of suitable methods to document LDL oxidation *in vivo*. The concentration of plasma or urinary isoprostanes are considered good markers of lipid peroxidation *in vivo*, particularly when assayed by gas chromatography–mass spectrometry.[34] For instance, isoprostane levels are higher in smokers and decline after cessation of this habit for 2 weeks. But levels do not increase acutely following smoking, so perhaps they reflect only medium- to long-term oxidative stress *in vivo*.[35] Our method quantifies the early oxidation products from all specific unsaturated fatty acids, not just arachidonic or docosahexaenoic acid.[29] The plasma concentration of hydroperoxy- plus hydroxy-fatty acids might be a valid marker of lipid peroxidation *in vivo*, provided samples are collected in the fasting state.[36]

The real problem with these methods to measure lipid peroxidation *in vivo* is that they are quite costly. Not surprisingly, one has turned to rather indirect measures of LDL oxidation and the measurement of the lag till the start of the rapid phase of lipid peroxidation is one of these. The lag phase until LDL oxidation is reduced in diabetics (type I and II) and in patients with established coronary heart disease.[14,16–18,20] These observations seem to justify the use of this *in vitro* method. Our results identify the need of a careful study design using LDL oxidation methods *in vitro*, if one is to avoid artefacts caused by seasonal variations. One also needs to remember that there are many factors that will determine the onset, the rate and the maximal lipid peroxidation of isolated LDL. Our study suggests that the amount of hydroperoxy- plus hydroxy-fatty acids in plasma lipids and hence LDL may be one of these. It is better to directly measure the factors that determine LDL susceptibility, because a reduced susceptibility in a particular condition may be due to the lack of antioxidants or increased lipid peroxides or both. And the precise knowledge of the reason for the unstable LDL immediately bears on the question

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how to avoid it. Furthermore, it is becoming increasingly apparent that these lipid peroxidation products exert biological effects, which might not at all be the same. This also argues against the use of a functional assay such as copper-mediated lipid peroxidation *in vitro*.

There is a well-established seasonal trend in myocardial infarction and coronary heart disease mortality,[37–39] presumably due to variations in atherosclerotic plaque stability and subsequent thrombosis during the year. It is tempting to speculate that "oxidative stress" is also increased during the winter months. Indeed, the higher plasma levels of hydroperoxy- and hydroxy-fatty acids could have originated from activated monocytes, atherosclerotic plaques in coronary or peripheral arteries. If so, the increased levels of oxidised lipids in the plaque increases the recruitment of monocyte/macrophages.[1] These cells determine the stability of the plaque.[40] We are unaware of seasonal variations in monocyte/macrophage infiltration into stable plaques.

In summary, there are seasonal variations in the susceptibility of LDL to lipid peroxidation and in oxidised plasma fatty acids in a group of affluent volunteers. Whether these variations are particularly pronounced in subjects at high risk of coronary disease and whether they can be linked to coronary events remains to be seen.

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