Antioxidants and resistance against oxidation of porcine LDL subfractions

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Abstract The objective of this study was to determine the level of antioxidants, the content of fatty acids and peroxidation products, and the resistance against oxidation of native porcine LDL_1 and LDL_2. There were no significant differences in the fatty acid distribution of both native low density lipoprotein (LDL) subfractions, which was similar to that of human LDL. The total amount of α- and γ-tocopherol of pig LDL was significantly lower than in human LDL, and β-carotene, lycopene, and retinyl esters were totally absent. Levels of thiobarbituric acid-reacting substances (TBARS) and lipid peroxides in freshly isolated pig LDL subfractions were below or only slightly above the detection limit. The susceptibility to oxidation of both LDL subfractions was investigated by addition of Cu^{2+} as prooxidant. The results show that pig LDL subfractions are much more susceptible to oxidation as measured by the duration of the lag phase preceding the onset of rapid lipid peroxidation. From the low content of vitamin E one would expect even much shorter lag phases. The possibility therefore exists that pig LDL contains additional, and as yet unidentified, antioxidants. **—Knipping, G., M. Rotheneder, G. Striegl, and H. Esterbauer.** Antioxidants and resistance against oxidation of porcine LDL subfractions. *J. Lipid Res.* 1990. **31:** 1965-1972.

Supplementary key words antioxidants • lipid peroxidation • pig low density lipoproteins

There are many well-established major risk factors for ischemic heart disease, i.e., hypercholesterolemia, which might cause endothelial cell damage (1) and thus may result in an atherosclerotic lesion. Several reports point to the cytotoxicity of low density lipoproteins (LDL) to various cell lines from human or animal origin under certain conditions (2,3). This cytotoxicity of LDL seems to result from lipoprotein oxidation. However, the molecular mechanisms of the oxidation processes are largely unknown, but it has been shown in vitro that peroxidation products are bound to LDL and thus modify the particle in a way that decreases the binding and uptake by the LDL receptor (4). This modified LDL becomes recognized by the scavenger receptor of macrophages with a concomitant accumulation of cholesteryl esters in these cells (5,6).

Pigs have been considered as a suitable animal model for investigation of human atherosclerosis, since the type and the location of spontaneous atherosclerotic lesions found in adult pigs are very similar to those seen in humans (7). Their lipoprotein profile resembles that of humans (8-10). As in the human, LDL is the principal transporter of cholesterol. Pig LDL may be separated into two subclasses, LDL_1 and LDL_2, which differ in size, lipid/protein ratio, and hydrated density. Both subfractions are physicochemically well characterized (11-13). Native porcine LDL, even when freshly prepared, is colorless and relatively unstable. In contrast, freshly prepared human LDL exhibits a strong yellow color primarily due to β-carotene, which disappears upon exposure of LDL to oxygen. One might expect LDL in pig serum to be mildly oxidized, since food for animals is normally bought in large amounts, and long and inappropriate storage of food may lead to its peroxidation. Additionally, some food may consist of already peroxidized waste products of nutritive substances. Peroxidized diets have been found to be toxic in animals, e.g., to cause sudden death with degenerative changes of heart muscle in the pig (14). It has been demonstrated that peroxides of arachidonic acid or other polyunsaturated fatty acids (PUFAs) are highly toxic when given orally (15).

The initiative of lipid peroxidation in LDL strongly depends on the antioxidant content (for review, see 16). In view of that and the recent hypothesis that oxidized LDL is involved in the formation of foam cells and fatty streaks, we analyzed pig LDL_1 and LDL_2 with respect to its content of PUFAs, antioxidants, endogenous peroxides, and its susceptibility towards oxidation.

Abbreviations: LDL, low density lipoproteins; LDL_1, Low density lipoprotein-1, buoyant density fraction 1.020-1.063 g/ml; LDL_2, low density lipoprotein-2, buoyant density fraction 1.063-1.090 g/ml; TBARS, thiobarbituric acid-reactive substances; HPLC, high performance liquid chromatography; GLC, gas-liquid chromatography; PUFAs, polyunsaturated fatty acids.

METHODS

Blood from individual pigs was immediately mixed with EDTA, and plasma was prepared by low speed centrifugation at 4 °C. EDTA (1 mg/ml) was present throughout all steps of preparation. Total LDL was prepared by sequential ultracentrifugation in the density range of 1.020–1.090 g/ml (9). The porcine LDL subfractions, LDL_1 and LDL_2, were further separated by density gradient ultracentrifugation (17). Both fractions were dialyzed against degassed 10 mM sodium phosphate buffer, 0.15 M NaCl, pH 7.4, for 24 h and stored until use at 4 °C under nitrogen in the dark.

Oxidation of low density lipoproteins

Pig LDL solutions were diluted with EDTA-free, oxygen-saturated 10 mM phosphate buffer, 0.15 M NaCl, pH 7.4 Oxidation was initiated by addition of freshly prepared $CuCl_2$ to LDL at room temperature. The final concentrations were 0.25 mg/ml LDL and 1.66 μM $CuCl_2$ (18).

Fluorescence measurements

Fluorescence spectra of native and oxidized LDL subfractions were recorded on a Hitachi F-4000 spectrofluorimeter. Excitation was performed at 360 nm and emission spectra were obtained from 380 to 520 nm. Band width was 10 nm for both excitation and emission. The instrument was standardized with quinine sulfate (0.1 μg/ml in 0.1 N H_2SO_4). The concentration of the native LDL fractions was 1 mg/ml, whereas the concentration of the oxidized samples was 0.25 mg/ml. For fluorescence measurement of the lipid phase and the apoprotein, the native and oxidized samples were delipidated with $CHCl_3$-CH_3OH 2:1 according to Folch, Lees, and Sloane Stanley (19). Lipids and apoproteins, obtained from 3 mg native LDL or 1 mg oxidized LDL, were dissolved in $CHCl_3$ and 3% aqueous SDS, respectively.

Analytical measurements

Unesterified and esterified cholesterol were measured with a test kit from Boehringer Mannheim, (FRG), triglycerides and phospholipids with test kits obtained from Biomerieux (Carbonniere les Bains, France). The protein content was determined according to Lowry et al. (20) using bovine albumin as a standard. Native and oxidized LDL samples were analyzed by SDS-gel electrophoresis using a gradient between 3.75% and 15% acrylamide. Whereas native LDL_1 and LDL_2 exhibited one single apoB band, a breakdown of apoB in the oxidized samples occurred resulting in low molecular weight products.

Fatty acids were analyzed as fatty acid methyl esters separated by capillary GLC on a 50-m glass column with CP Sil 88 (Chrompack) (21). The vitamin E content (22) of the LDL samples was determined from a aliquots of the lipid extracts (0.5 ml) as described earlier (21). HPLC analyses of lycopene and β-carotene were performed with detection at 450 nm (23) and that of retinyl esters at 326 nm (24). Thiobarbituric acid-reactive substances (TBARS) were determined as described (25). Lipid hydroperoxides were estimated according to El-Saadani et al. (26). Conjugated dienes were measured spectrophotometrically from the 234 nm absorbance assuming an ε value of 29,500 $M^{-1} \cdot cm^{-1}$ (18).

RESULTS

Chemical composition: fatty acids and antioxidants in porcine LDL_1 and LDL_2

Four preparations of LDL_1 and LDL_2, isolated from serum of different pigs, were used in this study to determine the chemical composition, i.e., protein and lipids (**Table 1**) and the individual fatty acids and antioxidants (**Table 2**). All LDL_1 samples had significantly higher lipid values ($P < 0.05$) than the corresponding LDL_2 except for triglycerides. The major fatty acids in LDL_1 and LDL_2 were linoleic (18:2) (40%), palmitic (16:0) (17–24%), and oleic acids (18:1) (18–20%), followed by stearic acid (18:0) (9–12%) and arachidonic acid (20:4) (4–9%). In one preparation LDL_1 and LDL_2 also contained traces (approximately 3% of total fatty acids) of 18:3, 22:5, and 22:6. The relative distribution of the individual fatty acids in a particular LDL_1 sample was always similar to that of the corresponding LDL_2 prepared from the same serum and there were no significant differences in the fatty acid distribution when the mean values of all analyses were compared (Table 2). For example, the relative percentage of PUFAs was nearly identical, namely 47.9 and 48.5 mol% in LDL_1 and LDL_2, respectively. The absolute amount of fatty acids (nmol/mg LDL) was somewhat lower in LDL_2, which is consistent with its lower cholesteryl ester and phospholipid content.

The major endogenous antioxidant detected in pig LDL was α-tocopherol; the average content in LDL_1 and LDL_2 was 0.73 and 0.94 nmol/mg LDL, respectively. Significant variations existed between the different preparations. In LDL_1 the α-tocopherol content, for example, ranged from 0.35 to 1.41 nmol/mg LDL; in LDL_2 the values were be-

TABLE 1. Chemical composition of native porcine LDL subfractions

Composition	LDL_1	LDL_2
Protein	24.6 ± 2.0	30.3 ± 2.9 ($P = 0.002$)
Free cholesterol	8.2 ± 0.4	6.6 ± 0.5 ($P = 0.025$)
Cholesterol esters	45.0 ± 1.7	40.9 ± 4.0 ($P = 0.05$)
Phospholipids	19.9 ± 1.9	17.1 ± 1.1 ($P = 0.025$)
Triglycerides	2.4 ± 0.4	5.2 ± 2.8 ($P = 0.1$)*

Values are presented as mean weight % ± standard deviation obtained from four different preparations. Statistical differences between LDL_1 and LDL_2 are shown by P values in parentheses. The data were analyzed by paired t-test;*, not significant.

TABLE 2. Fatty acids, antioxidants, and oxidation products in LDL_1 and LDL_2

	LDL_1	Range	LDL_2	Range
Fatty acids, nmol/mg LDL (n = 3)				
16:0	186.3 ± 36.3	158.0-227.2	163.2 ± 22.7	140.0-185.3
16:1	17.3 ± 19.0	0-38.4	20.1 ± 10.5	14-32.2
18:0	105.0 ± 20.2	82.5-121.9	98.7 ± 14.6	84.3-113.4
18:1	195.9 ± 26.7	166.4-218.4	181.2 ± 19.2	163.2-201.4
18:2	394.3 ± 16.5	375.3-405.2	365.9 ± 5.9	359.1-369.3
18:3	1.6 ± 2.7	0-4.7	1.6 ± 2.8	0-4.8
20:4	59.9 ± 24.2	39.5-86.7	59.5 ± 18.9	47.2-81.3
22:5	4.2 ± 7.2	0-12.5	4.6 ± 8.0	0-13.9
22:6	4.5 ± 7.7	0-13.4	4.4 ± 7.6	0-13.2
Total fatty acids	969.4 ± 35.3	929.3-995.9	899.2 ± 15.5	881.4-909.3
Total PUFA	464.4 ± 43.2	414.8-493.9	436.0 ± 19.5	416.4-455.4
Antioxidants, nmol/mg LDL				
α-Tocopherol (n = 4)	0.73 ± 0.49	0.35-1.41	0.94 ± 0.53	0.34-1.53
γ-Tocopherol (n = 4)	< 0.03	< 0.03-0.03	0.03	< 0.03-0.06
Carotene (n = 1)	< 0.02		< 0.02	
Lycopene (n = 1)	< 0.02		< 0.02	
Retinyl esters (n = 1)	< 0.02		< 0.02	
Oxidation products				
TBARS, nmol/mg LDL (n = 2)	0.70 ± 0.7	< 0.2-1.19	0.44 ± 0.33	< 0.2-0.67
Peroxides, nmol/mg LDL (n = 4)	< 2.0	< 2.0-2.38	< 2.0	
Fluorescence at 430 nm (n = 4) (relative intensity)	48 ± 12	37-59	80 ± 43	37-134

All values are presented as means ± standard deviation; n is the number of different LDL sample analyses.

tween 0.34 and 1.53 nmol/mg LDL (Table 2). γ-Tocopherol was present in detectable quantities only in two preparations; in the others it was below the detection limit, which was 0.03 nmol/mg LDL. Initially, we also tried to determine β-carotene, lycopene, and retinyl esters in one preparation. However, both carotenoids and retinyl esters were below the detection limit of 0.02 nmol/mg LDL. No further attempts were made to measure these antioxidants in the other LDL preparations. The absence of carotenoids in pig LDL is also consistent with the fact that none of the LDL subfractions showed a yellow color typical of carotenoid-containing lipoproteins.

The measurement of TBARS and lipid peroxides gave values below or only slightly above the detection limit, which was 0.2 nmol/mg LDL for TBARS and 2 nmol/mg LDL for lipid peroxides. Conjugated dienes were also undetectable in the lipid extract.

From previous studies of human LDL, it is known that oxidative processes generate fluorescent chromophores in the lipid and protein portion of the lipoprotein with emission maxima at about 430 nm (27). To further verify whether freshly prepared pig LDL is partly oxidized or not, fluorescence emission spectra (360 nm excitation, 380-520 nm emission) of the LDL samples and their lipid and protein moieties were recorded. A typical example for background-corrected spectra is shown in **Fig. 1**. Both LDL_1 and LDL_2 samples showed a broad diffuse emission between 400 and 500 nm with indications of maxima at 420, 435, and 460 nm. With one exception, the relative fluorescence intensity of LDL_2 was significantly higher compared to the corresponding LDL_1. The comparison of the fluorescence spectra of the lipid and protein fractions (Fig. 1) revealed that the fluorescence chromophores of pig LDL are mainly in the lipid moiety, exhibiting a fluorescence maximum at about 420-440 nm. The low fluorescence intensity of the various LDL subfractions was in agreement with the absence of detectable peroxidation products.

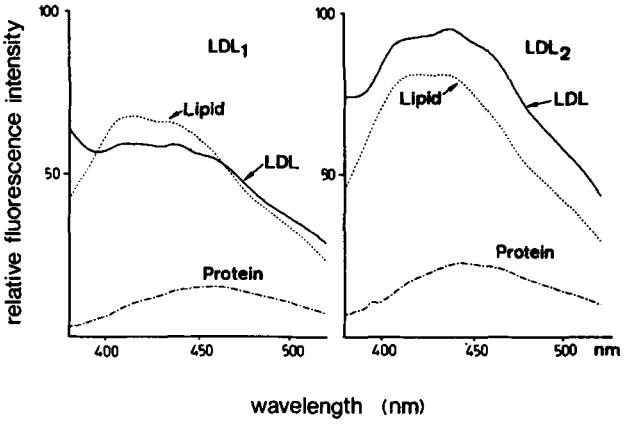

Fig. 1. Fluorescence spectra of LDL subfractions and their protein and lipid moieties. Spectra were recorded from 1.0 mg/ml native LDL_1 and LDL_2 from preparation III at excitation of 360 nm. LDLs were delipidated with $CHCl_3$-CH_3 OH 2:1 (v/v); the protein portion was dissolved in 3% aqueous SDS. The spectra are background corrected; (——) native LDL; (. . . .) lipids; (-·-·-) protein.

Copper-stimulated oxidation of LDL_1 and LDL_2

Many studies with human LDL revealed that Cu^{2+} ions act as strong prooxidants and initiate a lipid peroxidation process in LDL. In analogy to these studies, EDTA-free solutions of pig LDL subfractions (0.25 mg/ml) were supplemented with Cu^{2+} (1.66 μM) and incubated for 3 h. The oxidation process was stopped by the addition of excess EDTA (100 μM) and the fatty acids, antioxidants, lipid peroxidation products, and the fluorescence were determined (**Table 3**). All oxidized LDL samples were fully depleted of α- and γ- tocopherol as well as most of the PUFAs. The amount of the remaining linoleic acid was, on average, 16% of the original content in LDL_1 and 19% in LDL_2, respectively; the remaining arachidonic acid was only 2-3% and the PUFAs 18:3, 22:5, and 22:6 decreased to zero. The saturated (16:0, 18:0) and the monounsaturated (16:1, 18:1) fatty acids remained more or less unchanged. That Cu^{2+} has initiated a lipid peroxidation process in LDL_1 and LDL_2 became clearly evident by the strongly increased TBARS and peroxides. On an absolute scale the average loss of PUFAs in LDL_1 was 400 nmol/mg LDL and the oxidized LDL contained 200 nmol peroxides and 15.8 nmol TBARS; for LDL_2 the loss of PUFAs was 360 nmol, and 200 nmol peroxides and 17.1 nmol TBARS were present. Thus, in all cases the PUFA loss was significantly higher than the amount of peroxides present after 3 h, giving a molar yield of 0.51 peroxide/mol PUFA for LDL_1 and 0.56 for LDL_2.

The effect of incubation time on the concentration of peroxides in LDL_1 and LDL_2 was determined in preparations III and IV. A typical example is displayed in **Fig. 2**. After a variable induction period of approximately 30 to 60 min, the peroxide content rapidly increased, reaching a transient maximum value of about 220 nmol/mg LDL.

Cu^{2+}-stimulated oxidation of LDL subfractions resulted in a very strong increase of their fluorescence in the visible range of 400-500 nm. Typical spectra of LDL_1 and LDL_2 samples exposed to 1.66 μM Cu^{2+} for 3 h are shown in **Fig. 3**. The fluorescence maxima were at 435 nm. In order to determine the location of this newly generated fluorophore, the lipid and protein fractions of the oxidized LDL samples were isolated and their fluorescence spectra were measured again. As shown previously with human oxidized LDL (27), this measurement revealed that the 435 nm fluorophore is almost entirely associated with the protein moiety. This is in contrast to the situation in native LDL, where the lipid fraction shows the highest fluorescence. The lipids of oxidized LDL exhibited only a weak fluorescence with a rather broad diffuse maximum between 400 and 450 nm.

To further characterize some of the chemical properties of oxidized LDL_1 and LDL_2, UV spectra of the LDLs were continuously recorded under exposure to 1.66 μM Cu^{2+}. As already reported, a new absorption maximum occurs at 234 nm (18). This maximum is usually attributed to lipid hydroperoxides with conjugated carbon to carbon double

TABLE 3. Fatty acids, antioxidants, and oxidation products in LDL_1 and LDL_2 modified by Cu^{2+}-stimulated oxidation

	LDL_1	Range	LDL_2	Range
Fatty acids, nmol/mg LDL (n = 2)				
16:0	182.5 ± 23.0	166-189.9	170.5 ± 9.0	164.0-177
16:1	21.3 ± 7.9	15.7-26.9	22.3 ± 9.7	15.4-29.1
18:0	108.4 ± 1.6	107.3-109.5	106.2 ± 46.2	97.9-114.4
18:1	187.8 ± 19.3	174.1-201.4	196.7 ± 46.2	164.0-229.4
18:2	63.5 ± 1.6	62.3-64.6	74.8 ± 22.3	59.0-90.6
18:3	0	0	0	0
20:4	1.3 ± 1.8	0-2.5	1.8 ± 2.5	0-3.6
22:5	0	0	0	0
22:6	0	0	0	0
Total fatty acids	564.6 ± 13.3	555.2-574.0	572.2 ± 19.2	558.6-585.8
Total PUFA	64.7 ± 0.14	64.6-64.8	76.6 ± 19.8	62.6-90.6
Antioxidants, nmol/mg LDL (n = 4)				
α-Tocopherol	< 0.03		< 0.03	
γ-Tocopherol	< 0.03		< 0.03	
Carotenoids	< 0.02		< 0.02	
Oxidation products				
TBARS, nmol/mg LDL (n = 2)	15.8 ± 8.8	9.6-22.0	17.1 ± 6.6	12.4-21.8
Peroxides, nmol/mg LDL (n = 3)	202.4 ± 66.0	127.0-252.2	202.8 ± 43	155.3-240
Dienes, nmol/mg LDL (n = 4)	90.8 ± 29	51-119	72.8 ± 15	53-90
Fluorescence at 430 nm (n = 4) (relative intensity)	423 ± 165	219-623	419 ± 113	263-516
Lag phases (n = 4)	32 ± 15	18-53	38 ± 10	25-45

All values are presented as means ± standard deviation; n is the number of different LDL sample analyses.

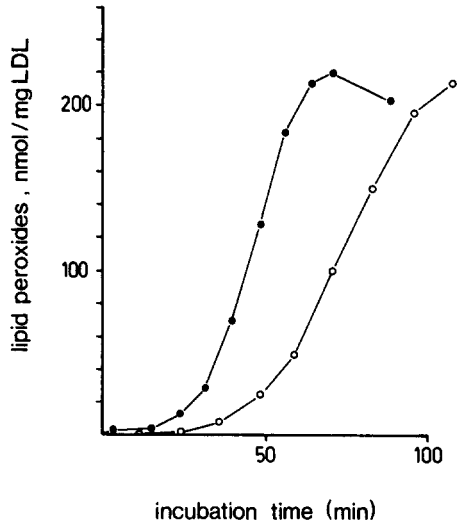

Fig. 2. Time profile of the change in peroxide content during Cu^{2+}-stimulated oxidation of porcine LDL subfractions. LDLs (0.25 mg/ml) form preparation III were in oxygen-saturated PBS; oxidation was initiated by addition of $CuCl_2$ to a final concentration of 1.66 μM. Peroxides were determined iodometrically; LDL_1 (●); LDL_2 (○).

bonds. Native, but not oxidized pig LDL samples, showed only a weak absorbance without a maximum at this wave length.

The continous measurement of the increase of the 234 nm absorbance with time was used to determine the kinetics of the Cu^{2+}-stimulated lipid peroxidation process in all LDL_1 and LDL_2 samples. **Fig. 4** shows typical examples of such experiments obtained for LDL preparations III and IV. The recorded curves always exhibited three distinct consecutive phases, namely an initial phase (lag time), where the absorbance only slowly increased; a second phase (propagation time) with a rapid and more or less linear increase of the 234 nm absorbance to a transient maximum value; and a third phase, where the absorbance slowly decreased again. Each LDL sample, however, showed its own characteristic behavior in respect to duration of the lag time and maximum absorbance. In the four LDL preparations, on average 90.8 ± 29 nmol dienes/mg LDL_1 and 72.8 ± 15 nmol/mg LDL_2 were formed, respectively. When the simultaneously measured peroxide values (Fig. 2) were plotted against the associated amount of dienes, a good linear relationship was obtained as shown in the insert of Fig. 4. However, in preparation IV the proportionality factors of LDL_1 and LDL_2 were clearly different and linearity only existed up to about 100 nmol peroxides/mg LDL.

A comparison of the vitamin E content of pig LDL with the associated lag phases showed a clear positive correlation with $r = 0.797$ (**Fig. 5**), with $y = 9.2125\,x + 17.519$. The standard error of the intercept was 6.15 and that of the slope 2.845.

DISCUSSION

We have determined the lipid composition, fatty acid composition, the content of antioxidants, and the resistance against oxidation in pig LDL_1 and LDL_2 subfractions. The lipid analyses (Tables 1 and 2) revealed that, except for triglycerides, the significantly higher lipid values in LDL_1 were the only noteworthy difference in the chemical composition of these LDL subfractions. The amount of antioxidants found in pig LDL was surprisingly low in comparison to human LDL. β-Carotene or lycopene, the major carotenoids in human LDL, were not detectable. Furthermore, the HPLC chromatograms did not show the presence of oxycarotenoids such as zeaxanthin or cryptoxanthin. Retinyl esters were also not present in detectable amounts. Concerning the latter, pigs belong to the group of mammals, e.g., humans, rabbits and rats, with negligible amounts of retinyl esters in plasma. In contrast, dogs contain an appreciable amount or retinyl esters in plasma associated almost entirely with LDL (28). The only detectable antioxidants in pig LDL were α-tocopherol and small amounts of γ-tocopherol. Moreover, the different LDL_1 and LDL_2 preparations showed significantly different α-tocopherol contents, ranging from 0.34 to 1.43 nmol/mg LDL. Expressed on a mol/mol basis (using a molecular mass of 2600 kDa for LDL_1 and 2000 kDA for LDL_2 a pig LDL particle contains only about one to four molecules α-tocopherol, whereas the vitamin E content of human LDL ranges from four to ten molecules/particle.

Human LDL, on average, contains per mg 2.77 nmol α- + γ-tocopherol, 0.35 nmol carotenoids (β-carotene + lycopene), and 0.57 nmol retinyl esters (29). The total content

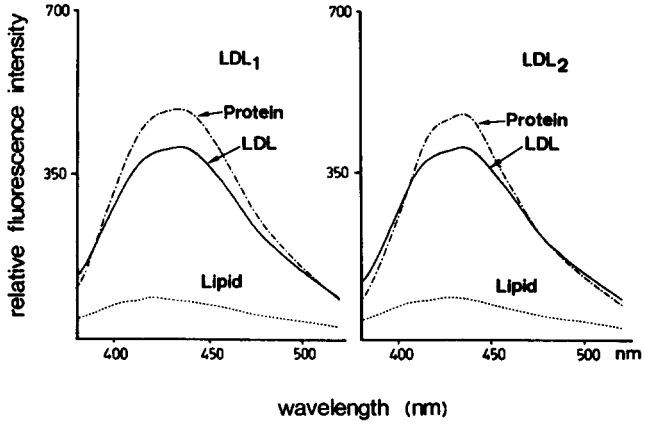

Fig. 3. Fluorescence spectra of oxidized LDL subfractions and their protein and lipid moieties; 0.25 mg/ml LDL_1 and LDL_2 from preparation III as used in Fig. 1 were oxidized for 3 h in the presence of 1.66 μM Cu^{2+}. Handling of samples and measurement conditions were the same as in Fig. 1. Spectra are corrected for solvent background; (——) oxidized LDLs; (. . . .) lipids; (–·–·–) protein. Note the different scale of the y-axis for native (Fig. 1) and oxidized samples.

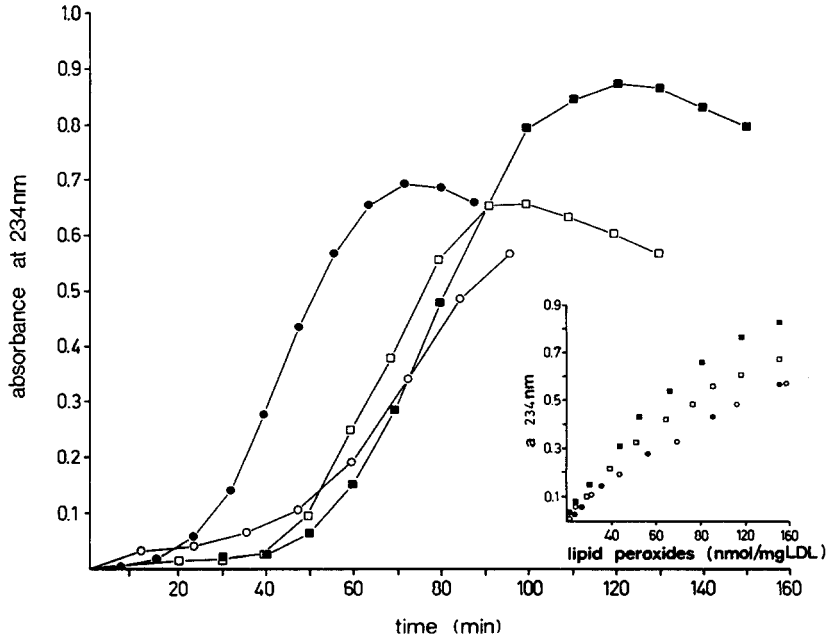

Fig. 4. Kinetics of Cu^{2+}-stimulated oxidation of pig LDL subfractions from preparations III and IV by monitoring the change of the 234 nm absorbance. Experimental conditions are the same as in Fig. 4, except that the change of the 234 nm absorbance was continuously monitored; (●) LDL_1; (○) LDL_2 from preparation III; (■) LDL_1; (□) LDL_2 from preparation IV. The insert shows the relationship of the increase of peroxides measured iodometrically (Fig. 2) and the increase of dienes by measuring the absorbance at 234 nm.

of antioxidants in pig LDL (0.85 nmol/mg) is, therefore, on average 4 times lower than that in human LDL (3.57 nmol/mg). Since the PUFA content of pig and human LDL is in the same range, in pig LDL the ration antioxidants/PUFA is, on average, 1:500 and in human LDL about 1:110. This would suggest that pig LDL is much more susceptible to oxidative modification than human LDL.

The lipid peroxidation process was continuously followed by measuring the increase of the 234 nm absorbance after exposure of LDL to Cu^{2+}. Fig. 4 shows that the rapid increase of the diene absorbance was always preceded by a lag phase, where the 234 nm absorbance only slowly increased. This lag phase can be ascribed to the action of chain-breaking antioxidants, which prevent a propagating chain reaction. It is thought that the duration of the lag phase, i.e., the oxidation resistance, depends on the content and effectiveness of such antioxidants. The lag phase of the pig LDL samples varied from 18 to 53 min. This is clearly shorter than that found for human LDL, which mostly gave lag phases in the range of 60 to 90 min (18). The shorter lag phase of pig LDL together with its low vitamin E content is, therefore, consistent with the assumption that vitamin E is an important factor for determining the oxidation resistance of LDL.

On a mol/mol basis, one vitamin E molecule per pig LDL particle can protect LDL on average for about 26 min against Cu^{2+}-stimulated lipid peroxidation. In human LDL, vitamin E seems to be less effective, since one vitamin E molecule per LDL particle protects only for about 4 min (30). Alternatively, the possibility should be considered that pig LDL contains other antioxidants in addition to vitamin E. This would be consistent with the intercept at zero vitamin E shown in Fig. 5, which suggests that a pig LDL molecule completely free of vitamin E possesses a residual oxidation

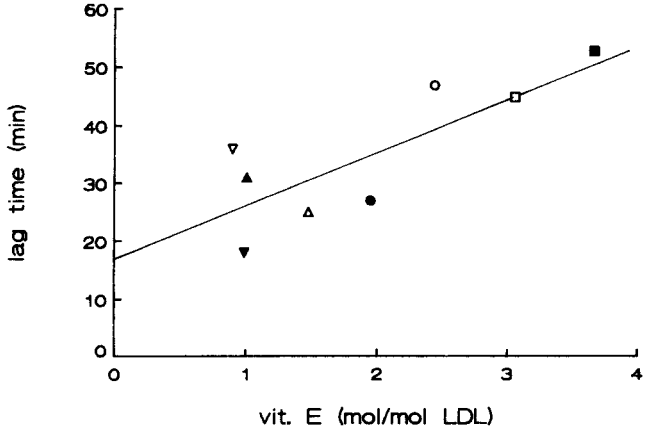

Fig. 5. Relationship between vitamin E content and lag time. The individual values of th different LDL preparations obtained for the lag time and the vitamin E content are plotted. Closed symbols, LDL_1, open symbols, LDL_2; (▲, △) preparation I; (▼, ▽) preparation II; (●, ○) preparation III; (■, □) preparation IV; y = 9.212 x + 17.5159; standard error of intercept and slope was 6.154 and 2.845, and *P* for intercept and slope was 0.029 and 0.0177.

resistance of about 17 min. It is known that ascorbate and also urate act as strong antioxidants in human LDL, and it could be that a small amount of these substances or other antioxidants are associated with isolated pig LDL and make it more resistant to oxidation.

No correlation whatsoever appears to exist between the lag time and PUFAs, whereas some trend of negative correlation (−0.58) was found between lag time and the ration of PUFAs to vitamin E.

In all likelihood the very low content of endogenous antioxidants in pig LDL is not due to oxidation processes that occur during LDL preparation, since TBARS, lipid peroxides, and conjugated dienes were in most pig LDL samples below the detection limit. Another rather sensitive parameter for oxidatively modified LDL is its fluorescence in the 400 to 450 nm range. Compared to human LDL (27) the fluorescence of the lipid moiety of native pig LDL, but not of the protein, in fact showed a higher fluorescence. However, with the present knowledge it is not clear whether this could indicate mild oxidative modifications of LDL having already occurred in vivo. It should be mentioned here that oxidized pig LDL (Fig. 3) revealed a fluorescence spectrum similar to native LDL (Fig. 1) though with a much higher intensity. Furthermore, in the case of oxidized LDL, the fluorescent chromophore is mainly associated with the protein moiety. This would rather suggest that the lipid fluorophore of native LDL is not related to oxidation processes. It seems, therefore, reasonable to assume that the low antioxidant content of pig LDL reflects the in vivo situation.

The fluorescence properties of oxidized pig LDL are in accordance with observations made on oxidized human LDL and indicate that the Cu^{2+}-stimulated oxidation led to a chemical modification of apoB, probably by binding aldehydes such as malonaldehyde (MDA) or 4-hydroxynonenal to lysine residues (27, 31, 32). We have determined MDA as TBARS in oxidized pig LDL, and found values between 9.6 and 22 nmol/mg LDL (Table 3). Compared to the loss of PUFAs or peroxides that were formed, this seems to be rather low. It must, however, be considered that TBARS can be formed mainly from 20:4 and 18:3, but hardly from the major PUFA, 18:2. The molar yield of TBARS (MDA equivalents) strongly correlates with the amount of 20:4 oxidized, and was in all experiments between 0.21 and 0.27, which means that after an oxidation time of 3 h about one MDA molecule is present per four molecules 20:4 oxidized.

We have previously determined the peroxide content in oxidized LDL by a method based on the modified TBA assay (33), and found in this case only about 12 nmol peroxides/mg human LDL (29). Later we questioned whether the peroxide content of oxidized LDL is indeed so low and measured the peroxides by an iodometric assay (26). In fact, this latter method gave at least ten times higher peroxide values in the range of 100–200 nmol/mg oxidized human LDL. Using this assay for pig LDL we obtained peroxide values in the range of 127 to 252 nmol/mg oxidized LDL.

We compared the discontinous measurement of peroxides and continuous measurement of dienes. Both methods gave comparable lag phases. For example, with the diene method the lag phases for LDL_1 from preparations III and IV were 27 and 53 min, respectively; those for LDL_2 were 47 and 45 min. Using the method for determination of peroxides, the lag phases for LDL_1 were 30 and 62 min, and those for LDL_2 49 and 53 min, respectively. However, the calculated ratio of the maximally formed peroxides and dienes was 2.33 ± 0.19 for LDL_1 ($n = 3$) and 2.79 ± 0.13 for LDL_2 ($n = 3$). These ratios indicate that, in oxidized LDL, peroxides are additionally present that are not the typical conjugated fatty acid hydroperoxides, i.e., -CH=CH-CH=CH-CH(OOH)-, and therefore are not detectable by measuring the 234 absorbance. Nevertheless, we prefer to use this method for the determination of the lag phase, since only 0.25 mg LDL is sufficient for a complete analysis, whereas 10 mg LDL is needed for the peroxide method.

In conclusion, the low content of antioxidants and therefore the higher susceptibility of pig LDL to oxidation may be one of the reasons for the early event of atherosclerosis in pigs. Our results further confirm the importance of vitamin E in preventing lipoprotein oxidation. However, the possibility exists that pig LDL contains additional not yet identified antioxidants, which may compensate to a certain degree for the low amounts of vitamin E.

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