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Formation of Free Radicals in Oxidation of Human Low-Density Lipoprotein. An EPR Spin Trapping Study

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The EPR spin trapping method with PBN as a spin trap was used to study the formation of free radicals in human low-density lipoprotein (LDL) upon ageing at 37 °C. The PBN associated radicals could be observed after 10 – 20 hours of incubation, depending on the pretreatment of LDL and the individual donor. The radicals observed in the first period of time (until about 20 – 25 hours of incubation) are related to the degradation products of PBN. After that, additional radicals associated with lipids are observed. The presence of EDTA does not prevent the process. The present experiments prove that some relatively fast oxidation process takes place in LDL incubated at physiological temperature. The process is mild, as judged from the essentially unchanged concentration of thiobarbituric acid-reactive substances (TBARS). An active role of the spin trap is not excluded.

INTRODUCTION

In living organisms, oxidatively modified low-density lipoprotein (LDL) behaves in a different way than native LDL. A major difference is due to the fact that oxidized LDL is not recognized by regular B/E LDL receptors, but by special scavenger receptors located predominantly on macrophages.[1] It is believed that the massive accumulation of lipids in macrophages, often called »foam« cells, is associated with early atherosclerotic lesions – fatty streaks.[1–3] It is generally accepted that both the initiation of lipid peroxidation and the propagation chain reactions are mediated by free radicals. However, the initiation step is not yet fully understood.[4] It is usually proposed that the first step

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is homolytic cleavage of preexisting lipid hydroperoxides of unsaturated fatty acids, catalyzed by transition metal ion complexes, with subsequent formation of lipid alkoxy and peroxy radicals.[4–6] These radicals stimulate the chain reaction of lipid peroxidation by abstracting further hydrogen atoms. The other possibility is the direct attack of some reactive species, like · OH and HO_2 · radicals, capable of abstracting hydrogen atoms from lipids.[4,5] These radicals could be formed in the iron-catalyzed Haber-Weiss reaction. The importance of the latter possibility has been questioned.[4]

Free radicals have already been directly observed in LDL during oxidation with EPR spectroscopy.[7–10] In the first EPR report,[7] the oxidation process was mild. It was sustained by continuous bubbling of oxygen through the buffer outside the dialysis bag containing the LDL sample. Later EPR reports[8–10] dealt with a severe metal ion – mediated oxidation. Here, we report the study on oxidation in a closed system, containing a limited amount of oxygen and without any purposely added initiator of oxidation. It will be shown that, even under these unfavorable conditions for oxidation, free radicals are formed. From the EPR spectra of spin trapped radicals, the structure of the radicals formed and the time course of their formation will be presented.

EXPERIMENTAL

Low-density lipoprotein was isolated from freshly drawn human plasma from 10 donors by step-wise density gradient ultracentrifugation within 1.020 – 1.063 g/cm^3. Throughout the process of isolation, LDL was protected from oxidation by EDTA. More details on the procedure can be found elsewhere.[11]

After isolation, the samples were first dialyzed against 0.02 M phosphate buffer (pH – 7.4) with EDTA (1 g/L) and stored at 4 °C before use. For the measurements, two sets of the LDL buffered solutions were prepared, one with and the other without EDTA. For the EDTA-free solution, EDTA was removed by dialysis against EDTA-free buffer. The LDL preparations were then concentrated, using AMICON B-15 concentrators, to a concentration of about 60 – 120 mg LDL/mL. For convenience, the LDL samples with EDTA are labelled LDL(+), and those without EDTA are LDL(–).

For the EPR measurements, the samples were prepared by mixing equal volumes of an LDL solution and the solution of the spin trap α-phenyl-*N-tert*-butylnitrone (PBN) (0.1 M) in the phosphate buffer (0.02 M). The samples were then placed in 100 μL micro pipettes and sealed.

Oxidation of the samples used for the EPR measurements was performed just by storing the samples in the EPR cavity at 37 °C. The EPR measurements were done as desired during the incubation period with a Varian E-109 spectrometer equipped with a Bruker temperature-control unit.

In order to test the general level of oxidation, the thiobarbituric acid reactive substances (TBARS) were measured. For that purpose, parallel samples were separately thermostated at 37 °C and tested for TBARS at regular intervals. TBARS were measured spectrophotometrically in a standard way.[12]

RESULTS

In no LDL preparation studied, free radicals could be detected immediately upon adding PBN to the lipoprotein solution. For freshly prepared samples, the incubation period of about 15 – 25 hours was needed for the appearance of the EPR spectra. For the samples stored for several weeks at 4 °C, this period was reduced to 8 – 15 hours. The EPR spectra typical of LDL(–) and LDL(+) at this early stage of the LDL oxidation are presented in Figure 1 (a and b) for the samples without EDTA (a) and with EDTA (b), together with the spectra for the phosphate buffer, incubated with PBN for a day (tracings c, d and e). The spectra clearly show the presence of two

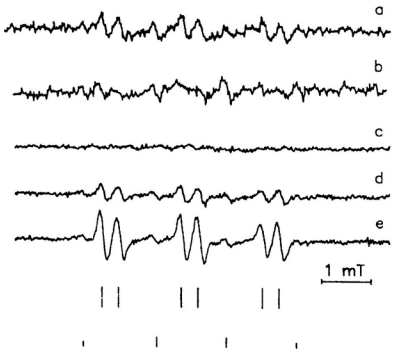

Figure 1. EPR spectra of the LDL solution (60 mg/mL) after incubation with PBN at 37 °C for 18 hours (a) LDL(–), (b) LDL(+). For comparison, the EPR spectra of similarly PBN-incubated buffers for 24 hours are shown (c) no EDTA added, (d) 1 mg/mL EDTA and (e) 2.5 mg/mL EDTA. Stick diagrams indicate the positions of a quartet pattern (solid bars) and a sextet pattern (dashed bars). The spectra were recorded at 37 °C with the microwave power of 10 mW and modulation amplitude of 0.15 mT.

types of radicals, obviously associated with degradation of the spin probe alone. One of the radicals, $PBN_1 \cdot$, is characterized by a quartet EPR pattern, indicated by the solid bars under the bottom spectrum. From the measured coupling constants, a_N = 1.46 mT and a_H = 1.39 mT, it is recognized to be *tert*-butyl aminoxyl.[13] The other radical, $PBN_2 \cdot$, characterized by the coupling constants a_N = 1.61 mT and a_H = 0.37 mT (dashed stick diagram in Figure 1) has not yet been assigned.[14]

The radicals associated with the PBN degradation products were not observed, or were barely observable after 24 hours of incubation of the EDTA-free buffer with PBN (Figure 1c). In the buffer with EDTA, the initial rate of production of both $PBN_1 \cdot$ and $PBN_2 \cdot$ radicals was larger for a higher concentration of EDTA (Figures 1d and e). The $PBN_1 \cdot$ radicals disappeared after about two days, and the $PBN_2 \cdot$ radicals remained stable for several days.

After incubation for about 48 hours, the EPR spectra of the LDL samples changed considerably and increased in intensity. Figure 2 shows the spectra for the oxidized LDL(–) samples of two different donors, A and B. The spectra clearly dem-

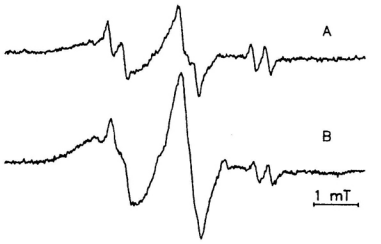

Figure 2. EPR spectra of the LDL(–) samples (40 mg/mL) of two different donors, A and B, after incubation for 48 hours. Other conditions are the same as in Figure 1.

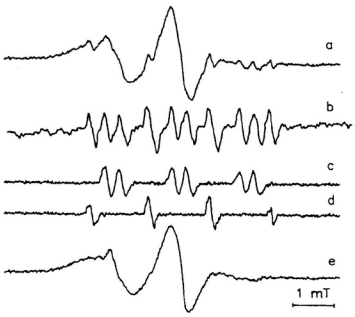

Figure 3. EPR spectra of an LDL(+) sample (donor C) recorded after 48 hours of incubation (top curve) and of the filtrate (curve b), together with three constituent spectra of the LDL pattern. The constituent spectra are assigned to $PBN_1 \cdot$ (d), $PBN_2 \cdot$ (c) and the lipid radical (e). Other conditions are the same as for Figure 1.

onstrate the presence of the two radicals associated with the degradation of PBN, together with additional radicals.

In order to characterize the additional radicals, obviously associated with LDL, the aqueous phase of an LDL sample was extracted by N_2 pressure dialysis and the EPR spectra of the filtrate were recorded. Figure 3 shows the EPR pattern of a thermally treated LDL(+) sample of another donor (C) (curve a) and the filtrate (curve b).

Further, the experimentally obtained spectra for LDL were decomposed into the component spectra with the aid of a computer. The EPR pattern shown in Figure 3a is easily decomposed into three constituent elementary spectra: a quartet assigned to $PBN_1 \cdot$, (curve d), a sextet assigned to $PBN_2 \cdot$ (curve c) and a difference spectrum (curve e). The elementary spectra of $PBN_1 \cdot$ and $PBN_2 \cdot$ were derived from the PBN-incubation buffers (Figure 1).

The difference spectrum (Figure 3e) belongs to the trapped lipid radicals of the L · and/or LO · type, presumably of LOO · origin.[15] It is likely that both of these radicals are present. For PBN-trapped LO ·, the typical values of the coupling constants are: a_N = 1.39 mT and a_H = 0.20 – 0.25 mT, for L · associated with PBN the values a_N = 1.48 mT and a_H = 0.28 – 0.35 mT are considered appropriate.[16,17] These coupling parameters cannot be verified from the EPR spectra of highly immobilized radicals, like the spectrum in Figure 3e. That spectrum is essentially the same as that reported by Kalyanaraman and coworkers,[9] attributed to the carbon-centered PBN-trapped lipid radical. The coupling constants deduced in earlier work from the more resolved spectra of the same radicals in more viscous medium, a_N = 1.49 mT and a_H = 0.22 mT,[9] do not perfectly fit to a standard PBN-trapped L · radical.[16,17] However, the oxygen-centered PBN adducts, characterized by even more different parameters,[14,17] are not expected to survive such a long incubation without a supply of new oxygen, like in the present system.[14]

Also, our results with unsaturated fatty acids undoubtedly show that, in the aerated samples in a closed chamber and in the deareated samples after storage at elevated temperatures, only the L · spin adducts are observed.[17]

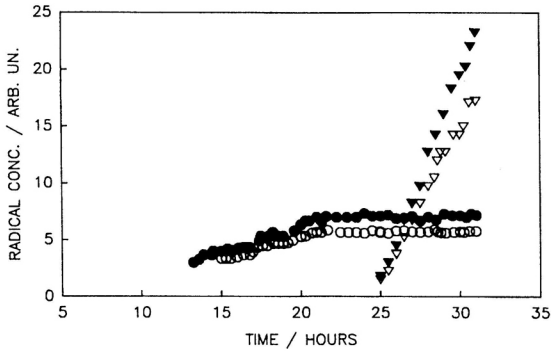

Figure 4. Relative concentration of the radicals in an LDL(–) sample (40 mg/mL) (full symbols) and LDL(+) (open symbols) as a function of incubation time. Circles indicate the relative concentration of the $PBN_2 \cdot$ radicals and triangles those of the PBN-trapped lipid radicals, deduced from the intensity of the integrated EPR spectra.

After an initial increase with the time of incubation, the PBN degradation radicals either disappear ($PBN_1 \cdot$) or very slowly decrease ($PBN_2 \cdot$), and the lipid PBN adducts increase. A typical behaviour of $PBN_2 \cdot$, the predominant PBN-degradation radicals, and the lipid radicals in the LDL(–) and (LDL(+) samples with time is shown in Figure 4. It is interesting to note that the concentration of the $PBN_2 \cdot$ radical ceases to increase when the lipid radicals appear.

The amount of thiobarbituric acid-reactive substances did not change significantly during the incubation of the LDL preparations at 37 °C in a closed chamber. Standard values of TBARS for freshly prepared samples varied from 0.02 to 0.05 nmol MDA/mg LDL, and increased most for the factor of 2 during the incubation period of 2 days.

The experiments at room temperature and at 45 °C show qualitatively the same behaviour. The only marked difference is a slower rate of radical formation at room temperature and a faster rate at 45 °C.

DISCUSSION

In most of the oxidation studies on human plasma lipoproteins so far, the transition metal ions were present as an essential prooxidant component, either in the cell culture or in the buffer.[18,19] So is also the EPR spin trapping work of Kalyanaraman's group[8–10] related to the metal-ion mediated free radical production. There is only one report on the direct EPR evidence of the free radical formation in the LDL oxidation that is not initiated or supported by purposely added transition metal ions or other initiators.[7] The present work is an extension of our previous study inasmuch as we now impose further restrictions on the oxidizing capacity of the LDL system and use the computer-assisted decomposition of the experimentally obtained EPR spectra into constituent patterns, which can be resolved and assigned. In contrast to the previous study,[7] here we use a closed system. In such a system, the amount of oxygen that can be used is greatly reduced.

Also, the LDL preparation is carried out with good quality deionized water and, in addition, a chelating agent EDTA is added. It is shown that, even under these conditions, the radicals associated with the lipid domain of LDL are readily formed although the TBARS, as a generally used index of oxidation, do not indicate any breakdown of the polyunsaturated fatty acids.

More information on the events in the lipid core could be obtained by a thorough chemical analysis, which remains the subject of our next study.

It is not quite understood what species are responsible for the initiation of oxidation. Possible mechanisms have recently been reviewed,[4] but neither of the possibilities seem to be applicable here. However, it is observed that in the initial stage of the oxidation (incubation at 37 °C), the radicals associated with the degradation products of PBN are formed. After a certain time period (about 20 hours), the lipid radicals appear at the expense of the growth of the $PBN_1 \cdot$ and $PBN_2 \cdot$ radicals, indicating a possible role of the spin trap in the radical production.

The EPR pattern of the PBN-trapped L · radical (Figure 3e in the present work and Figure 4A in Ref. 9) points to much more restriction for the radical motion in the present system than in neat lipids.[17,20] The reason might lie in the fact that the present system is less fluid, particularly the surface part of LDL, and that in the present system the lipids and, consequently, the PBN-trapped lipid radicals (phospholipids and cholesteryl esters) are larger molecules.[21]

The role of EDTA appears contradictory. From the studies on pure buffers, EDTA seems to be required for the formation of $PBN_1 \cdot$ and $PBN_2 \cdot$. In contrast, studies of the LDL preparations demonstrate that almost equal amounts of the radicals associated with PBN degradation are formed in LDL(+) and LDL(–) (Figure 4). Only the ratio of these two radicals is different: in LDL(–) $PBN_1 \cdot$ is barely observed. Also, the formation of lipid radicals is independent of the presence of EDTA. It is possible that a certain amount of EDTA is present in all of our LDL samples. Dialysis against EDTA-free buffer, intended for removal of EDTA, might not be successful in completely removing EDTA bound to the LDL particles.

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SAŽETAK

Stvaranje slobodnih radikala u oksidaciji ljudskog lipoproteina. Istraživanje metodom EPR

Dubravka Krilov, Carol M. Herak-Kramberger, Tatjana Ukrainczyk i Janko N. Herak

Metodom EPR spektroskopije, uz pomoć spinske stupice PBN promatrano je stvaranje radikala u ljudskom lipoproteinu male gustoće (LDL) pri starenju na 37 °C. Radikali vezani na PBN opažaju se nakon 10 – 20 sati inkubacije, ovisno o donoru i predpovijesti uzorka. Radikali opaženi u prvomu vremenskom periodu (do 20 – 25 sati) odnose se na produkte raspada PBN. Nakon toga perioda opažaju se i radikali povezani s lipidima. Prisutnost EDTA ne sprečava odvijanje procesa. Ova opažanja pokazuju da se na fiziološkoj temperaturi odvija relativno brz proces oksidacije LDL. Kako se u tom procesu gotovo ne povećava koncentracija tvari što reagiraju sa tiobarbiturnom kiselinom (TBARS), autori zaključuju da je proces oksidacije blag. Aktivna uloga spinskih stupica nije isključena.