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EPR Kinetic Studies of the LDL Oxidation Process Driven by Free Radicals

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Oxidation is widely assumed to play a causal role in the pathogenesis of atherosclerosis. Oxidative modification of low density lipoprotein (LDL) can be followed by analyzing the lag phase of the conjugated diene formation at 234 nm in LDL exposed to Cu^{2+}. This procedure is restricted to isolated LDL fractions. To make this assay applicable to different biological systems, the present paper introduces a method to determine the time course of lipid peroxidation by measuring the EPR signal intensity and thereby the concentration of the radicals formed. Stable radical spin adducts were generated using the spin trap PBN (N-tert.-butyl-α-phenylnitrone) and were detected by EPR spectroscopy. Comparing the specific formation of radicals and the generation of conjugated dienes as measured by UV absorbance revealed analogous lag, propagation and decomposition phases.

Keywords: Antioxidants, copper, EPR, LDL, PBN, spin trapping

Abbreviations: EDTA, ethylenediamine tetraacetic acid; EPR, electron paramagnetic resonance; LDL, low density lipoprotein; PBN, N-tert.-butyl-α-phenylnitrone; PBS, phosphate-buffered saline; PUFA, polyunsaturated fatty acid; UV, ultraviolet; α-TocH, α-tocopherol

INTRODUCTION

Oxidation is widely thought to be a major event in the pathogenesis of atherosclerosis. It has been hypothesized that oxidative modification of LDL is related to free radical production.[1,2] Different forms of chemically modified LDL have been shown to lead to the accumulation of cholesterol in macrophages. This unrestricted uptake of modified LDL has been reported to be mediated by nonspecific scavenger receptors on the macrophage surface.[3] In contrast, uptake of unmodified LDL by LDL receptors is strictly regulated.[4]

Modification of LDL can be achieved by incubation of LDL with specific cells (endothelial cells, smooth muscle cells, macrophages) or by incubation with traces of transition metals (copper, iron).[5,6] Interestingly, both copper and iron levels are significantly increased in atherosclerotic, compared to healthy, arterial walls.[7,8] Epidemiologic studies have shown a positive

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correlation between serum copper and iron levels and risk of cardiovascular disease, supporting the theory that LDL oxidation may be mediated by transition metals.[9–11]

Different assays have been used to determine oxidized derivatives like lipid hydroperoxides, aldehydes or short chain hydrocarbons (ethane, pentane).[5,12–14] Esterbauer *et al.*[15] introduced a method to measure the rate and degree of Cu^{2+}-induced LDL oxidation by continuously monitoring the absorbance at 234 nm. The procedure allows to determine the amount of early products of lipid peroxidation, the conjugated dienes. This method is suitable to show effects of pro- and antioxidant components in the LDL particles, but it is not possible to measure the influence of water-soluble (enzymatic and non-enzymatic) antioxidants originally contained in the investigated plasma sample. In addition, the isolation of LDL by ultracentrifugation might lead to the transfer of tocopherols from one lipoprotein class to another thus leading to artificial conditions.

EPR spectroscopy is the only physical technique that can detect and identify short lived free radicals in a reacting system. Kalyanaraman *et al.*[16] have shown that LDL-derived lipid radicals can be measured directly using the spin trap technique. They demonstrated that incubation of LDL in a PBS buffer with PBN generates stable radical spin adducts. Thomas *et al.*[17] obtained several time-dependent EPR spectra on this basis. Although this study has shown a slight increase of the EPR signal intensity, detailed EPR kinetic data of the PBN–LDL radical adduct formation are not available. Furthermore, to monitor the radical-driven oxidation of LDL it is not suitable to add PBN to the reaction solution, since PBN is a potent antioxidant.[16] Kinetic data would necessarily differ when PBN is present. Finally, due to the fact that the radical adduct of PBN is rotationally restricted in LDL, it is more convenient for quantitative measurements to use the better resolved EPR spectrum by extraction of the lipophilic substance in an organic solvent as obtained with, e.g., hexane.

This report presents a method to follow the Cu^{2+}-induced oxidation of LDL by measuring the formation of PBN radical adducts employing EPR. Using the EPR spectroscopy could make the assay applicable to different biological systems. Kinetic EPR measurements may allow the determination of the lag phase without being restricted to purified LDL solutions. With this technique, the kinetics of radical formation, i.e. observation of lag phases can be determined. Comparing the results of EPR and UV measurements, the relationship between the existence of free radicals and the production of conjugated dienes in the copper catalyzed lipid peroxidation process can be investigated.

MATERIALS AND METHODS

Chemicals and Solutions

KBr, NaCl, KH_2PO_4, Na_2HPO_4, EDTA, $CuSO_4$, $MgSO_4$ and hexane were of highest grade commercially available and were purchased from Merck, Germany or Aldrich, USA. Enzymatic assays (colorimetric test Chol MPR 1) for the determination of cholesterol concentration were from Boehringer Mannheim. PBN was obtained from Sigma, USA and was stored at −18°C under an atmosphere of nitrogen. Unless indicated otherwise, all buffer solutions pH 7.4 were stored for 12 h over Chelex 100 resin, 100–200 mesh (sodium form, 6 g/l) from Bio-Rad, USA to remove divalent metal ions and were deoxygenated by bubbling argon gas through the solution.

LDL Separation

LDL was isolated from human plasma by ultracentrifugation as described by Chung *et al.*[18] Fresh whole blood was obtained by venipuncture from a single human volunteer and the EDTA–plasma was immediately used to isolate LDL. A discontinuous NaCl/KBr density gradient was

formed by adjusting the density of the plasma to 1.3 g/ml with KBr and layering 10 ml under 24 ml normal saline ($d = 1.006$ g/ml). Sorvall Ultra-crimp-Tubes (QTY 25, 35 ml) were placed in a Sorvall TV-850 vertical rotor and ultracentrifuged in a Sorvall OTD 65B ultracentrifuge (339791g, 50 000 rpm, 10°C, 3 h). The LDL band was obtained with a syringe needle and was characterized by polyacrylamide gel electrophoresis.[18] EDTA-containing LDL solutions were stored under an atmosphere of argon in the dark at 4°C.

In Vitro Oxidation of LDL

LDL oxidation with Cu^{2+} was carried out according to Esterbauer *et al.*[15,19] LDL, purified just prior to use, was freed from EDTA by gel-chromatography with Econo Pac 10 DG columns purchased from Bio-Rad, USA. The LDL concentration was calculated from the data of total cholesterol 2200 mol/mol LDL, 100 mg/dl cholesterol $\equiv$ 0.26 mmol/dl and the LDL molecular weight of 2.5×10^6 Da. LDL was diluted with oxygenated phosphate buffered saline (PBS: 160 mmol NaCl, 10 mmol sodium phosphate buffer) to give a final concentration of 0.25 mg/ml LDL. At 30°C, oxidation of LDL was induced by addition of a freshly prepared aqueous $CuSO_4$ solution to a final concentration of 1.66 µmol/l. To verify that EPR and UV measurements are results of a Cu^{2+}-induced lipid peroxidation experiments were performed by inclusion of an excess of EDTA (5 mmol/l) in the LDL reaction mixture either prior to the addition of Cu^{2+} or at the end of the reaction.

UV Measurements

The kinetics of the LDL oxidation process were followed in a Beckman DU 640 spectrophotometer by continuously monitoring the increase of the absorbance at 234 nm in intervals of 5 min in a 1 cm quartz cuvette.

EPR Measurements

EPR spectra were recorded at 293 K using a Bruker ESP 300E spectrometer, equipped with a Bruker TE_{102} (ER 4102ST) cavity, operating at 9.6 GHz with a 100 kHz modulation frequency. A microwave power of 6.3 mW and a modulation amplitude of 1 G were typically employed. The EPR measurements were performed by extracting 1000 µl of the aqueous LDL solution with 1000 µl freshly distilled hexane containing 5 mg PBN. After vortexing for 90 s, the sample was microcentrifuged (16000g, 22°C, 60 s) in a 2 ml Eppendorf tube. The upper hexane phase was removed and dried by vortexing with anhydrous $MgSO_4$ (15 s). The hexane extracts were transferred to a capillary cell and deoxygenated by bubbling argon gas through the solution. The total radical concentration was determined by measuring the peak to peak amplitude of the middle field signal.

RESULTS

Figure 1 shows the EPR spectra of PBN radical adducts from an LDL solution at indicated times after the initiation with 1.66 µmol/l $CuSO_4$. The typical PBN spectrum consisting of a triplet of doublets due to the nitrogen and β-H coupling ($A_{N(1)} = 13.61$ G and $A_H = 1.94$ G) of the spin adduct can be seen at each time point. Formation of the radical adducts of PBN was only observed when a freshly prepared $CuSO_4$ solution was added to an oxygenated phosphate buffered saline solution (pH 7.4) containing 0.25 mg/ml LDL. Radicals were extracted from aliquots of the reaction mixture by hexane and were stable in this solution for at least one hour. No such increase of PBN radical adducts was observed without copper (Figure 2(a)) or when an excess of EDTA over copper was added prior to the addition of the metal (data not shown). The addition of EDTA to the LDL incubation immediately before the extraction procedure had no influence on the radical formation. Particularly

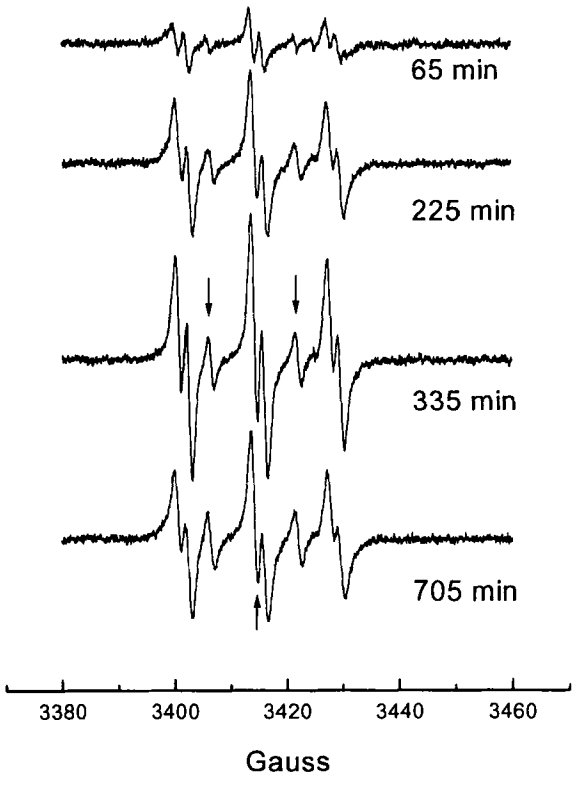

FIGURE 1 EPR spectra of PBN radical adducts resulting from an oxygenated phosphate buffered saline solution containing 0.25 mg/ml LDL. Oxidation was initiated by addition of $CuSO_4$ to give a final concentration of 1.66 µmol/l. The EPR spectra were obtained at 65, 225, 335 and 705 min after the addition of Cu^{2+}. EPR measurements were performed as described in Materials and Methods. Instrumental conditions: modulation amplitude: 1 G, receiver gain: $2*10^5$, conversion time: 81.92 ms, time constant: 81.92 ms, number of scans: 8. Arrows (↓) indicate a second spectral component as described in the text.

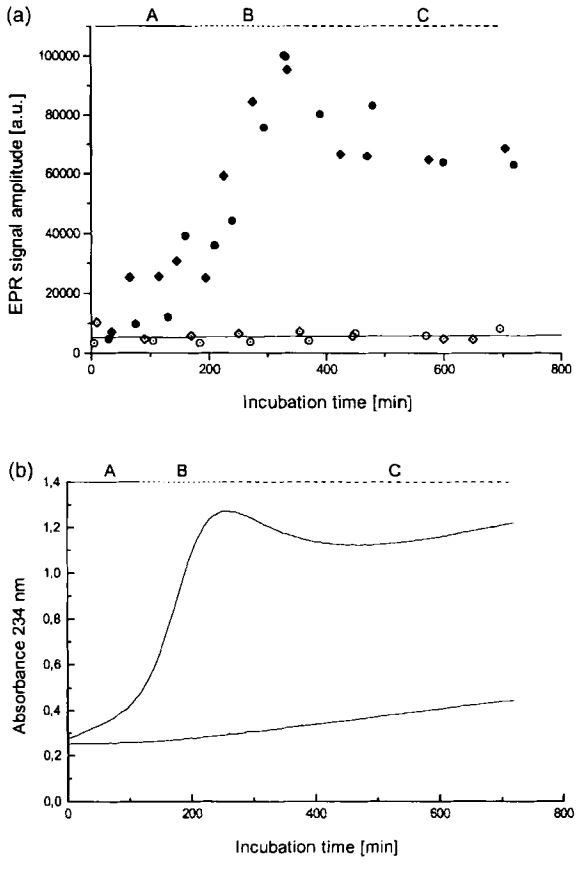

FIGURE 2 (a) EPR spectroscopic kinetic measurements of the LDL oxidation in the presence of Cu^{2+}. (●, ◆) represent data obtained from a single donor at different days. The EPR middle field signal amplitude was recorded over a time period of 720 min. Linear regression was used to fit the control samples (⊙, ◇) which were not exposed to Cu^{2+}. Experimental conditions are as given in the legend to Figure 1. (b) LDL oxidation (0.25 mg LDL/ml PBS) followed by measuring the absorbance at 234 nm in intervals of 5 min in a 1 cm quartz cuvette. Oxidation was initiated by addition of $CuSO_4$ to give a final concentration of 1.66 µmol/l. The lower trace represents the control, where no Cu^{2+} was added. Measurements were performed in duplicate. Averages are shown.

striking are the two signals (see arrows (↓)) in the middle field region of the spectrum, indicating that a second PBN adduct overlaps the six line spectrum. Obviously the high and low field peak (↓) are consistent with peaks derived from a second triplet with a coupling constant of $A_{N(2)} = 7.78$ G. Due to the superposition of the two triplets the total radical concentration was determined by measuring the peak to peak amplitude of the middle field signal ((↑) see Figure 1).

The time course of the PBN radical adduct formation can be clearly divided into distinct phases (A, B and C) (Figure 2(a)). A lag phase (A) during which the radical concentration did not or only slightly increased was observed up to approximately 170 min of the incubation time. The second phase (B) is characterized by an exponential increase in the EPR signal leading to a maximal level. The third phase (C) shows a decrease and subsequent stabilization of the PBN radical adduct concentration.

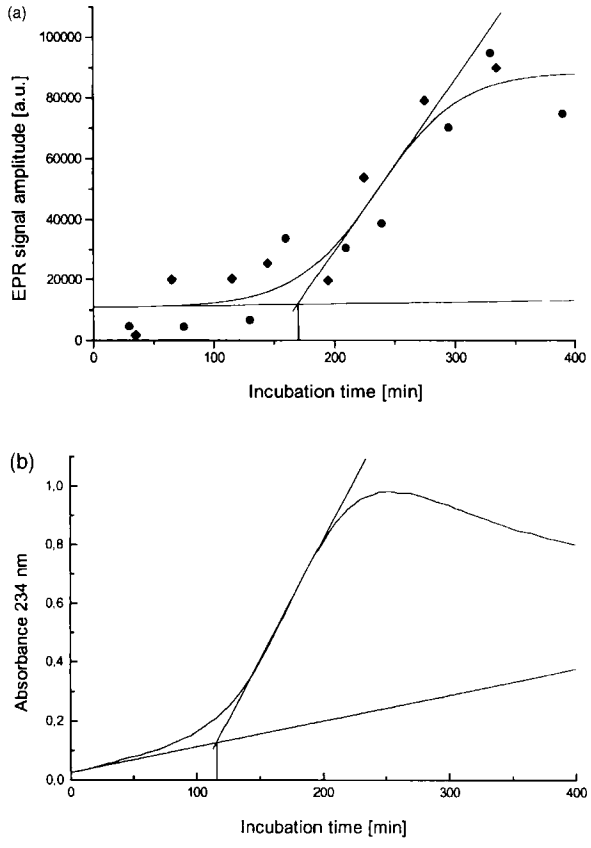

FIGURE 3 (a) Determination of the lag phase by EPR measurements. Data (●, ◆) of intrinsic oxidation processes were obtained by subtracting the control data (no Cu^{2+} added) from the experimental data (Cu^{2+} added) (see Figure 2(a)) and plotting the difference. The curve represents a sigmoidal fit to all points. The lag phase (170 min) was determined graphically as the intercept of the tangents of the sigmoidal oxidation curve calculated with a computer program (MicroCal Origin version: 3.5, Microcal Software, Inc.). (b) Determination of the lag phase by UV measurements. Data were obtained by subtracting the control from the oxidation process as in (a) except that the UV absorbance data (see Figure 2(b)) was used. The lag phase (115 min) was determined graphically as described in the legend to (a).

To further evaluate the correlation of the radical concentration with the amount of conjugated dienes, it was essential to compare the EPR and UV measurements. Although much more scatter was evident in the observation made by EPR, both methods showed very similar lag-, propagation- and decomposition-phases (compare Figure 2(a) and (b), phases A, B and C). The lag phase as determined for the EPR and UV measurements in Figure 3(a) and (b) (EPR: 170 min, UV: 115 min) revealed a difference of 55 min that may or may not be due to the scattering of the EPR data. A similar difference was observed in the times needed for the formation of maximal PBN spin adduct concentrations (EPR maximum: 330 min, UV maximum: 250 min) compared to the formation of maximal amounts of conjugated dienes. Measurements performed over a time period of 900 min revealed no significant changes in the EPR signal amplitude (from 575 to 900 min), but a continuous increase in the UV absorbance was observed in this range due to decomposition products[15] (data not shown).

DISCUSSION

The time profile for the formation of PBN radical adducts as obtained by EPR measurements in this study (Figure 2(a)) revealed three distinct phases (A, B and C) of the Cu^{2+}-induced LDL oxidation. The lag phase (A) during which the EPR signal hardly increased may suggest that the antioxidants of LDL are consumed without liberating substantial amounts of free radicals. In the second phase (B), which is characterized by a strong increase in the amount of PBN radical adducts, net formation of lipid hydroperoxides seems to occur. Decreasing concentration of free radicals towards a slightly lowered plateau (C), points out the end of the radical-driven process.

A proposed mechanism for the lipid peroxidation of the PUFA is presented in Figure 4. Initiation of the copper-dependent oxidative modification of LDL requires reduction of Cu^{2+} to Cu^{+} as a first step. The role of Cu^{2+} as the prooxidant was proven by experiments with addition of EDTA to the LDL reaction mixture. Previous studies[20–22] have shown that α-tocopherol plays a triggering role in the LDL oxidation, especially when contaminating metal ions are removed by using Chelex 100 resin as carried

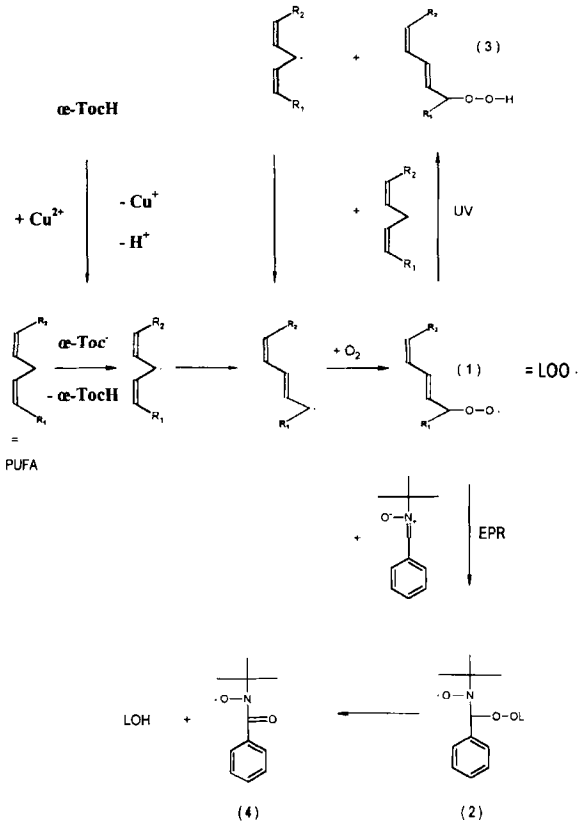

FIGURE 4 Proposed mechanism of the copper-mediated oxidation of LDL.

out here. It has been suggested that lipid peroxidation observed in these systems is initiated by a direct interaction between α-tocopherol and Cu^{2+} with generation of Cu^{+}. Reaction of the α-tocopheroxyl radical with the activated methylene group between the double bonds leads to a carbon-centered lipid radical. The alkyl radical reacts rapidly with oxygen yielding a peroxyl radical (LOO$^{\bullet}$ (1)). Abstraction of a hydrogen atom from another PUFA by the peroxylradical (1) serves to continue the chain reaction. The products are lipid hydroperoxides (LOOH (3)) which can directly be determined by measuring the amount of conjugated dienes.[15,23] The reaction of PBN with peroxyl radicals (LOO$^{\bullet}$ (1)) yields PBN radical adducts (2) that display typical six line EPR spectra.

Previous studies have demonstrated the scavenging of the LDL lipid-derived radical by PBN.[16,17] Kalyanaraman *et al.*[16] have reported the formation of the PBN–LDL radical adduct in ethanol with the EPR hyperfine coupling constants of $A_N = 14.9$ G and $A_H = 2.2$ G. These spectral parameters are not consistent with those reported in the present study ($A_{N(1)}$=13.61 G and $A_H = 1.94$ G). However, Yamada *et al.*[24] showed the trapping of LOO$^{\bullet}$ radicals with PBN at room temperature in benzene with coupling constants close to this work. PBN radical adducts of peroxyl radicals generated from the hydroperoxide of methyl linoleate gave hyperfine splitting constants of $A_N = 13.44$ G and $A_H = 1.63$ G (for a review see Mottley and Mason[25]).

Elimination of alcohol (LOH) from (2) could generate a new product with an α-keto function (4) that may be responsible for the additional triplet overlapping the normal set of three doublets (see Figure 1). Janzen and Blackburn[26] observed a spectrum of two nitroxides after mixing di-*n*-butyl-lead dichloride with PBN. This data was interpreted as one nitroxide from the spin adduct giving the six line spectrum superimposed on another spectrum ($A_N = 7.67$ G) assigned to benzoyl-tert.-butyl-nitroxide. It was concluded that the latter coupling constant could only be consistent with products with an α-keto function. Further examples have been described.[27–29] The deviation of the coupling constant for (4) with A_N=7.78 G in this case is possibly caused by the solvent hexane used here, whereas Janzen and Blackburn used benzene in their EPR experiments.

Comparison of the kinetic data obtained from EPR or UV spectroscopy revealed analogous lag-, propagation- and decomposition-phases. The similar increase of PBN radical adducts and the amount of conjugated dienes demonstrates that LDL oxidation is a radical-driven process. The slight deviation in the lag phases and the shift in the propagation phases to later times relative to the formation of conjugated dienes may be caused by a dimerization of carbon-centered

radicals at the beginning of the propagation phase, which could explain the formation of conjugated dienes without the determination of PBN radical adducts by EPR.

Radicals were extracted at times from aliquots of the reaction mixture by hexane and were stable in this solution for at least 1 h. Hence, the method is robust enough to allow for routine measurements as it does not require a time protocol once the radical adduct has been extracted. Unfortunately, in this study a quantitative estimate of the radical concentration was not possible, because the exact composition and concentration of the various fatty acids on which a quantitation would have to be based was not known. We simply used LDL preparations from one human donor. However, quantitation seems feasible within certain limits.[30]

The formation of conjugated dienes can be followed by continuously monitoring the increase in absorbance at 234 nm as introduced by Esterbauer *et al.*[15] several years ago. This experimental determination of the lag phase, representing the susceptibility of LDL to oxidative modification, is restricted to purified LDL preparations. Although Thomas *et al.*[17] obtained several time-dependent EPR spectra of the PBN–LDL radical adduct, the relationship between the total radical concentration and the amount of conjugated dienes formed in this reaction, as well as the respective kinetics, remained unknown. The present work describes a method to investigate the first steps of free radical-driven lipid peroxidation by measuring the EPR signal intensity as a function of time. This method will allow for measurements of free radical formation, e.g., in blood plasma, cell suspensions or tissue homogenates under different pro- and antioxidative conditions.

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