FEBS Letters 501 (2001) 42–46 FEBS 25036

Ceruloplasmin as low-density lipoprotein oxidase: activation by ascorbate and dehydroascorbate

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Received 30 May 2001; revised 9 June 2001; accepted 12 June 2001

First published online 26 June 2001

Edited by Barry Halliwell

Abstract The ability of ceruloplasmin (Cp) to oxidize low-density lipoproteins (LDL) in the presence of water-soluble antioxidants was investigated and a reaction mechanism proposed. Ascorbate strongly enhanced LDL oxidation, but only after its rapid consumption. Dehydroascorbate enhanced Cp-mediated LDL oxidation even more strongly. Lipid-soluble antioxidants and water-soluble peroxides did not show noticeable activation. However, loading of LDL with lipid hydroperoxides increased the initial oxidation rate. We conclude that Cp mediates a localized redox cycle, where reduction of Cp-Cu^{2+} is effected by water-soluble reductants and reoxidation by liposoluble hydroperoxides.

Key words: Low-density lipoprotein; Ceruloplasmin; Plasma antioxidant; 15-Lipoxygenase; Lipid peroxidation; Atherosclerosis

1. Introduction

Ceruloplasmin (Cp; $M_r = 132$ kDa, reviewed in [1,2]) is a blue plasma protein (300 μg/ml approx., 2–3 times more in the acute phase of inflammation) carrying about 95% of total circulating copper. Its biological function is still not quite clear, although a number of activities have been described, including copper transport, oxidation of organic amines [3], ferroxidase activity, glutathione peroxidase activity [4] and antioxidant activity in lipid peroxidation processes [5]. Prooxidant activity of Cp has been previously reported in several instances, such as the oxidation of DNA [6] and low-density lipoprotein (LDL), both by intact Cp [7], and after release of redox-active copper either by low pH [8] (e.g. at sites of inflammation) or by nitric oxide/peroxynitrite [9]. Surprisingly, Cp has long been regarded as an exclusively antioxidant enzyme, because the prooxidant activity of intact Cp was overlooked due to the copurification of a metalloproteinase in Cp preparations, leading to cleavage of Cp and concomitant loss of prooxidant activity [10].

The crystal structure of Cp [11] confirmed the presence of six copper ions. However, Cp contains seven copper ions in total, one of which is a prooxidant in lipid peroxidation reactions and can be easily removed by chelation [12].

Cp has been reported to function as and to have structural similarities to 'proper' ascorbate (AscH) oxidases. In the presence of oxygen, a large excess of AscH immediately reduced the copper sites and kept them in a reduced state [13].

The oxidation of LDL is assumed to play an important role in the development of atherosclerosis (for a review see [14]). However, it is still unclear how LDL can be oxidized in vivo; the presence of high concentrations of water-soluble antioxidants in plasma and endothelial lining fluid [15,16] (urate (UA), AscH) has been shown to synergistically prevent oxidation of lipoproteins under in vitro experimental conditions [17], where initiation of LDL oxidation was induced by free transition metal ions or reactive oxygen and nitrogen species.

Transition metal ions are probably responsible for a large part of the oxidized lipids and proteins found in atherosclerotic plaques. Indeed, copper and iron have been detected in lesion areas in sizeable quantities [18]. However, it has been argued that metal ions might not exist in free form, but are bound to proteins, such as transferrin or Cp. Consistently, ferroxidase activity, indicative of Cp, was detected in atherosclerotic plaques [19], besides immunohistochemical detection of the Cp protein [20].

Here, we show that the oxidation of LDL is not merely possible in the presence of water-soluble plasma antioxidants, but that the efficient oxidation of LDL by Cp depends on the presence of reducing cosubstrates and we propose a mechanism for lipoprotein oxidation by Cp.

2. Materials and methods

All materials were of analytical grade or better, and from Sigma or Merck (Vienna, Austria), unless specified otherwise. AscH was from Loba (Fischamend, Austria). Rabbit reticulocyte 15-lipoxygenase (15-LOX) was a kind gift from Prof. Hartmut Kühn (Hospital Charité, Berlin, Germany).

2.1. Preparation of human Cp

Cp was prepared according to [21]. After overnight dialysis against bidistilled water, Cp was lyophilized and dissolved in phosphate-buffered saline (PBS), aliquoted and stored at −20°C. In accordance with published procedures, Cp purity was checked by SDS–PAGE, showing the preparation to be >95% intact. The ratio of Cp-specific and aromatic amino acid residue absorbance, A_{610}/A_{280}, as another indicator of purity, was always >0.047. Cp concentrations were determined from its absorbance at 610 nm, with $E^{1\%}_{610} = 0.68$ [7].

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Abbreviations: LDL, low-density lipoprotein; Cp, ceruloplasmin; EDTA, ethylenediamine tetraacetic acid; LL-CL, low-level chemiluminescence; 15-LOX, 15-lipoxygenase; UA, urate, uric acid; AscH, ascorbate, ascorbic acid; DHA, dehydroascorbate, dehydroascorbic acid

0014-5793/01/$20.00
PII: S0014-5793(01)02623-0

2.2. Preparation of LDL

LDL was prepared from pooled ethylenediamine tetraacetic acid (EDTA) plasma of healthy volunteers of both sexes by ultracentrifugation in a single-step discontinuous gradient in a Beckman NVT65 rotor, as described previously [22]. Prior to use, LDL was stored in an evacuated glass vial under argon at 4°C for a maximum of 2 weeks. LDL concentration was determined from its total cholesterol content, using the CHOD-PAP enzymatic test kit (Boehringer-Mannheim, Germany), assuming a molar mass of LDL of 2.5 MDa and a cholesterol content of 32.2 wt%.

2.3. LDL oxidation

Prior to oxidation of LDL, EDTA and KBr were removed by gel filtration as described [23]. Unless indicated otherwise, oxidation was performed at 37°C by addition of a 50 μM stock solution of Cp to a 0.3 μM solution of LDL, both in PBS (160 mM NaCl, 10 mM Na-phosphate, pH 7.4), to give a final concentration of 2.25 μM Cp. All experiments were performed at least in duplicate.

2.4. Low-level chemiluminescence (LL-CL) measurements

LL-CL was measured in a LUCY I luminometer (Anthos Labtech Instruments, Salzburg, Austria) equipped with a single-photon-counting photomultiplier (sensitivity 300–700 nm) as described [24]. Integration time was 90 s, measurements were performed in a white microplate. LL-CL is a method for continuously monitoring lipid peroxidation far superior to UV measurement of conjugated dienes (giving similar results) in the presence of substances absorbing strongly in the ultraviolet spectral range. The intensity of LL-CL is proportional to the square of the oxidation rate [24].

2.5. Loading of LDL with α-tocopherol

LDL was loaded with α-tocopherol as described [25]. Briefly, EDTA plasma was incubated with ethanolic α-tocopherol (250 mM, 1% of total plasma volume) for 2 h at 37°C under argon in the dark, and LDL was isolated subsequently as described above.

2.6. Determination of AscH and α-tocopherol

AscH was determined according to [26] using samples stabilized with 5% *meta*-phosphoric acid and detected electrochemically (Ag/AgCl 0.6 V, HP1049A detector). α-Tocopherol was determined by HPLC and fluorescence detection after extraction of LDL, as described [27].

3. Results

3.1. Activation of Cp by AscH

LL-CL was monitored during Cp-mediated LDL oxidation in the presence of AscH (2.25 μM Cp, 0.3 μM LDL, 0–100 μM AscH); similar results were obtained by measuring conjugated diene formation (not shown). However, due to high UV absorbance all further experiments were performed using LL-CL. Saturable activation of Cp by AscH was observed (Fig. 1A shows one representative experiment), LL-$CL^{1/2}_{max}$ vs. [AscH] was fitted by a hyperbola and the activation constant, K_a, determined to be 19.1 ± 4.5 μM (Fig. 1B).

Increasing AscH concentration both slightly reduced the lag phase of oxidation and increased the maximal oxidation rate. The lag phase at 20 μM AscH was only about one-third of the lag phase in the absence of the reductant.

We also tested UA, the major water-soluble antioxidant in human plasma, and found a similar, albeit much weaker activation (K_a = 59.8 μM; data not shown). Addition of 50 μM H_2O_2, in order to check for an effect on the Cu^{2+}/Cu^{+} redox cycle, could not activate Cp in LDL oxidation, nor was AscH-enhanced Cp oxidation inhibited (data not shown).

In order to ensure that it was not release of free copper from Cp which led to extensive LDL oxidation we also separated LDL and Cp by a dialysis membrane, which effectively prevented LDL oxidation even in the presence of elevated concentrations of AscH (not shown).

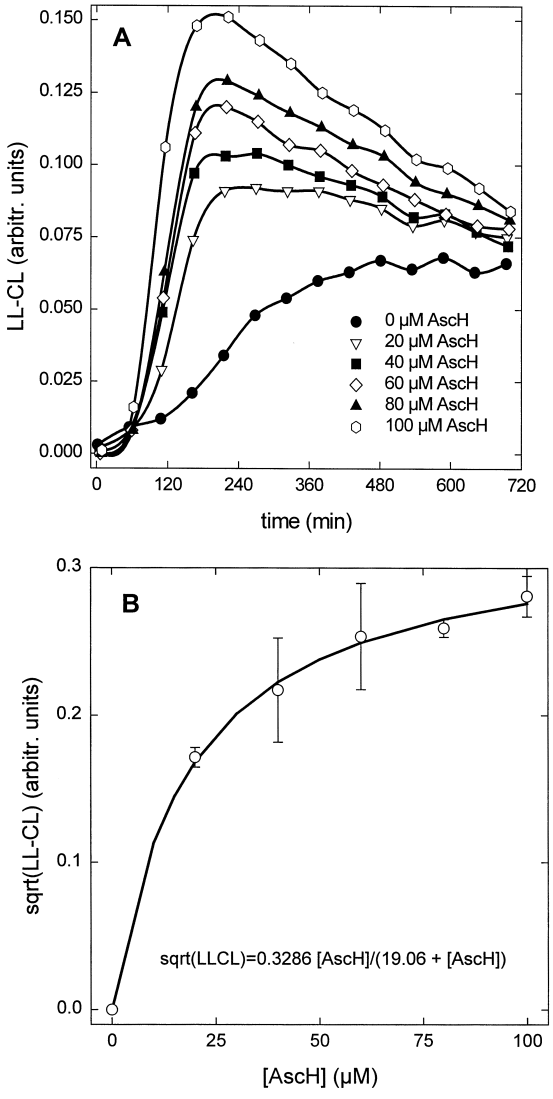

Fig. 1. Enhancement of Cp-mediated LDL oxidation by AscH. LDL (0.3 μM) was oxidized in the presence of 2.25 μM Cp and the progress of oxidation monitored by LL-CL. A: One representative series of experiments with AscH = 0–100 μM. B: A plot of the square root of ($LL\text{-}CL_{max,\ 20–100\ \mu M\ AscH} - LL\text{-}CL_{max,\ 0\ \mu M\ AscH}$) vs. [AscH] and the fitted hyperbola of the saturation kinetics. The square root of LL-CL corresponds to the oxidation rate.

3.2. Consumption of AscH and α-tocopherol during Cp-mediated LDL oxidation

During Cp-mediated LDL oxidation (1.5 μM Cp, 0.1 μM LDL), AscH was consumed prior to α-tocopherol (Fig. 2A), within about 60 min. AscH spared α-tocopherol, consistent with findings for Cu^{2+}-induced LDL oxidation [28]. Loading of LDL with α-tocopherol did not change the lag phase (not shown) and the lifetime of α-tocopherol (Fig. 2B), nor was there an increase in maximal LL-CL intensity observed, indicating that α-tocopherol did not redox-activate Cp (not shown).

3.3. Activation of Cp by dehydroascorbate (DHA)

Figs. 1 and 2 suggest that maximal activation of Cp by

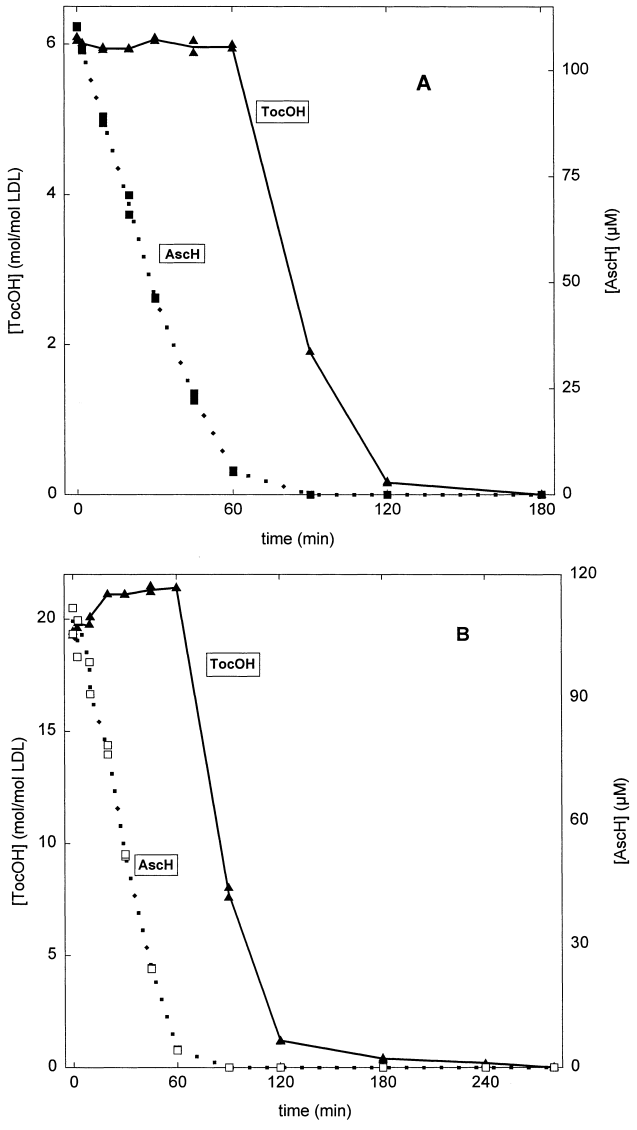

Fig. 2. Consumption of AscH and α-tocopherol during Cp-mediated LDL oxidation. A: Native LDL (0.1 μM) was incubated with 1.6 μM Cp and 100 μM AscH and aliquots of the oxidation mixture were used to assay for AscH and α-tocopherol. B: Assay conditions as in A, except that instead of native LDL, LDL loaded with α-tocopherol was used.

AscH occurs only after complete oxidation of AscH by Cp. Hence, we tested whether DHA, the two-electron oxidation product of AscH, was able to activate Cp. Fig. 3A shows one representative experiment and Fig. 3B shows the fit of the hyperbola of a saturation kinetics to the plot LL-$CL_{max}^{1/2}$ vs. [DHA], similar to Fig. 1. From this plot, the K_a for DHA was calculated to be 14.0 ± 2.0 μM, which is even lower than for AscH, indicating a higher affinity of DHA for the active site of the LDL oxidase function of Cp.

3.4. Incubation of LDL with 15-LOX

To assess the influence of lipid hydroperoxides, 0.3 μM LDL was incubated for 30 min at 37°C with increasing concentrations of 15-LOX, in the presence of 1 mM Ca^{2+}, 50 μM AscH and 300 μM UA, to better approach the physiological situation and meet the requirements of 15-LOX for binding to LDL (Fig. 4). While the maximal oxidation rate remained unchanged at increasing LOX concentrations, the initial oxidation rate increased concentration-dependently.

4. Discussion

Based on the results given above a mechanism for Cp-mediated LDL oxidation in the presence of water-soluble reductants is proposed. The redox-active entity is the labile Cu^{2+} (Cp-Cu^{2+}), which has been described by Ehrenwald et al. [7]. For oxidation of LDL, intact Cp must bind directly to the lipoprotein.

4.1. Water-soluble antioxidants as activators of Cp-mediated LDL oxidation

Water-soluble antioxidants which are present in human plasma in substantial concentrations, e.g. AscH (50–100 μM) or DHA (and UA, 300–400 μM), can act as enhancers

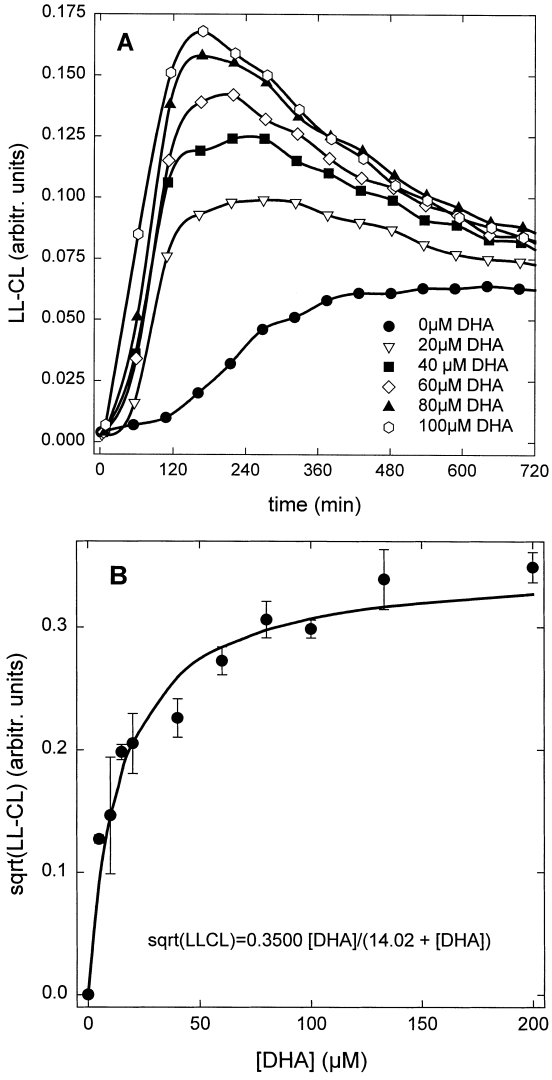

Fig. 3. Enhancement of Cp-mediated LDL oxidation by DHA. LDL (0.3 μM) was oxidized in the presence of 2.25 μM Cp and the progress of oxidation monitored by LL-CL. A: One representative series of experiments with DHA = 0–100 μM. B: A plot of the square root of ($LL\text{-}CL_{max,\ 5-200\ \mu M\ DHA} - LL\text{-}CL_{max,\ 0\ \mu M\ DHA}$) vs. [DHA] and the fitted hyperbola of the saturation kinetics. The square root of LL-CL corresponds to the oxidation rate.

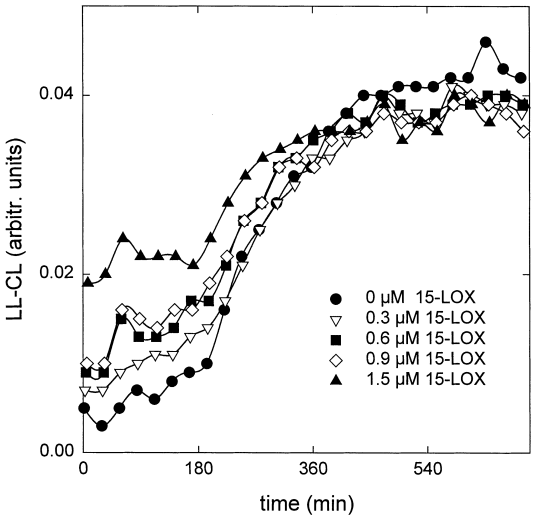

Fig. 4. Synergistic effects of 15-LOX and Cp in LDL oxidation. LDL (0.3 μM, 50 μM AscH, 300 μM UA) was incubated for 30 min with the indicated concentrations of 15-LOX. Oxidation was then started by addition of 2.25 μM Cp.

of lipid peroxidation by Cp, instead of exerting their 'traditional' role in prevention of LDL oxidation. This is even more remarkable in light of the previous finding that AscH is able to prevent prooxidant effects which might otherwise occur in the presence of reductants during metal-catalyzed LDL oxidation [17].

Lipid-soluble antioxidants, namely α-tocopherol, were not able to enhance Cp-mediated LDL oxidation, even at elevated concentrations, i.e. after loading of LDL with the antioxidant. In the case of LDL oxidation mediated by free Cu^{2+}, redox activation by α-tocopherol has been shown to be the trigger for oxidation, in the absence of lipid hydroperoxides [27,29]. However, the time course of antioxidant consumption indicates that AscH is directly oxidized by Cp, protecting α-tocopherol in LDL as long as it is not fully consumed. The strong activation of Cp-mediated oxidation clearly occurs after the consumption of AscH which indicates that not AscH, but an oxidation product may be the true activator.

4.2. DHA activates Cp

Using DHA instead of AscH in the oxidation assays, an even stronger activation of Cp ensued, suggesting that it may actually be DHA (or its hydrolysis products, e.g. 2,3-diketogulonic acid) which is responsible for activation. This is corroborated by its K_a value, which is actually even lower than the K_a for AscH. DHA has been shown previously to become prooxidant in LDL oxidation once the endogenous α-tocopherol content of LDL is exhausted [17,30,31]. Hence, it appears that the AscH oxidase activity of Cp first depletes AscH but in turn provides DHA as another powerful reductant to the prooxidant copper.

4.3. Saturable activation of Cp by reductants

The observation that different reductants show a saturable effect on LDL oxidation by Cp and lead to different K_a values (and also to different maximal rates: LL-CL_{max} of UA is only 30% that of AscH and DHA (not shown)) indicates an quasi-enzymatic mechanism, where the reductants act as cosubstrates. Moreover, the reductive step apparently occurs exclusively from the aqueous side of the bound enzyme, because lipid-soluble reductants such as α-tocopherol are not able to activate Cp, either in the absence or in the presence of water-soluble reductants (Fig. 2). To complete a prooxidant redox cycle of the Cu^{2+} responsible for LDL oxidase activity [12], there must be a reoxidation step after reduction. Addition of H_2O_2 proved ineffective in this, neither enhancing Cp-mediated LDL oxidation nor inhibiting it, which might have been the case if it competed e.g. with lipid hydroperoxides. This points to a separation of the oxidation and reduction reactions of Cp in such a way that reduction occurs from the aqueous phase exclusively, and reoxidation of the Cu^{+} takes place from the lipid phase – which is also evident from loading of LDL with lipid hydroperoxides using 15-LOX (Fig. 4).

Acknowledgements: This work was supported by the Austrian Science Foundation, Projects SFB00709 (to P.M.A. and S.F.) and SFB00711 (to J.S.F.), and the European Union, BIOMED2 CA Project PL963191. The authors are indebted to Riccardo Albertini, R. Jörg Schaur and Ruth Prassl for helpful discussion. The expert technical assistance of Ms. Alexandra Zirngast is gratefully acknowledged.

References

[1] Fox, P.L., Mukhopadhyay, C. and Ehrenwald, E. (1995) Life Sci. 21, 1749–1758.
[2] Fox, P.L., Mazumder, B., Ehrenwald, E. and Mukhopadhyay, C.K. (2000) Free Radical Biol. Med. 28, 1735–1744.
[3] Zaitsev, V., Zaitseva, I., Papiz, M. and Lindley, P.F. (1999) J. Biol. Inorg. Chem. 4, 579–587.
[4] Kim, I.G., Park, S.Y., Kim, K.C. and Yum, J.J. (1998) FEBS Lett. 431, 473–475.
[5] Al-Timini, D.J. and Dormandy, T.L. (1977) Biochem. J. 168, 283–288.
[6] Kim, R.H., Park, J.E. and Park, J.-W. (2000) Free Radical Res. 33, 81–89.
[7] Ehrenwald, E., Chisolm, G.M. and Fox, P.L. (1994) J. Clin. Invest. 93, 1493–1501.
[8] Lamb, D.J. and Leake, D.S. (1994) FEBS Lett. 338, 122–126.
[9] Swain, J.A., Darley-Usmar, V. and Gutteridge, J.M.V. (1994) FEBS Lett. 342, 49–52.
[10] Ehrenwald, E. and Fox, P.L. (1994) Arch. Biochem. Biophys. 309, 392–395.
[11] Zaitseva, I., Zaitsev, V., Card, G., Moshkov, K., Bax, B. and Ralph, A. (1996) J. Biol. Inorg. Chem. 1, 15–23.
[12] Mukhopadhyay, C.K., Mazumder, B., Lindley, P.F. and Fox, P.L. (1997) Proc. Natl. Acad. Sci. USA 94, 11546–11551.
[13] Musci, G., Bonaccorsi di Patti, M.C. and Calabrese, L. (1993) Arch. Biochem. Biophys. 306, 111–118.
[14] Esterbauer, H., Schmidt, R. and Hayn, M. (1997) Adv. Pharmacol. 38, 425–456.
[15] Sevanian, A., Davies, K.J.A. and Hochstein, P. (1991) Am. J. Clin. Nutr. 54, 1129S–1134S.
[16] Dabbagh, A.J. and Frei, B. (1995) J. Clin. Invest. 96, 1958–1966.
[17] Abuja, P.M. (1999) FEBS Lett. 446, 305–308.
[18] Lee, F.-Y., Lee, T.-S., Pan, C.-C., Huang, A.-L. and Chau, L.-Y. (1998) Atherosclerosis 138, 281–288.
[19] Swain, J. and Gutteridge, J.M.C. (1995) FEBS Lett. 368, 513–515.
[20] Hollander, W., Colombo, M.A., Kirkpatrick, B. and Paddock, J. (1979) Atherosclerosis 34, 391–405.
[21] Farver, O., Bendahl, L., Skov, L.K. and Pecht, I. (1999) J. Biol. Chem. 274, 26135–26140.
[22] Ramos, P., Gieseg, S.P., Schuster, B. and Esterbauer, H. (1995) J. Lipid Res. 36, 2113–2129.
[23] Puhl, H., Waeg, G. and Esterbauer, H. (1994) Methods Enzymol. 233, 425–441.
[24] Albertini, R. and Abuja, P.M. (1998) Free Radical Res. 29, 75–83.
[25] Puhl, H., Waeg, G. and Esterbauer, H. (1993) Methods Enzymol. 233, 425–441.

[26] Bui, M.H., Sauty, A., Collet, F. and Leuenberger, P. (1992) J. Nutr. 122, 312–316.
[27] Abuja, P.M., Albertini, R. and Esterbauer, H. (1997) Chem. Res. Toxicol. 10, 644–651.
[28] Kagan, V.E., Serbinova, A.A., Forte, T., Scita, G. and Packer, L. (1992) J. Lipid Res. 33, 385–397.
[29] Kontush, A., Meyer, S., Finckh, B., Kohlschütter, A. and Beisiegel, U. (1995) J. Biol. Chem. 271, 11106–11112.
[30] Stait, S.E. and Leake, D. (1996) Biochem. J. 320, 373–381.
[31] Otero, P., Viana, M., Herrera, E. and Bonet, B. (1995) Free Radical Res. 27, 619–626.