Presence of CuZn superoxide dismutase in human serum lipoproteins

P. Mondola[a,*], M. Bifulco[b,c], R. Serù[a], T. Annella[a], M.R. Ciriolo[d], M. Santillo[a]

[a] *Dipartimento di Neuroscienze e della Comunicazione Interumana-Sez. Fisiologia, Via S. Pansini 5, 80131 Naples, Italy*
[b] *CEOS, CNR, Dipartimento Biol. Patol. Cell. Mol., Università degli Studi di Napoli 'Federico II', Via S. Pansini 5, 80131 Naples, Italy*
[c] *Dipartimento Med. Sper. Clin., Università di Catanzaro, Catanzaro, Italy*
[d] *Dipartimento di Scienze Biomediche, Università di Chieti 'Gabriele D'Annunzio', Chieti, Italy*

Received 3 December 1999; received in revised form 10 January 2000

Edited by Guido Tettamanti

Abstract It has previously been demonstrated that CuZn-superoxide dismutase (SOD) is secreted by several human cell lines. This suggests that the circulating enzyme derives from both hemolysis and peripheral tissues as a result of cellular secretion. In the present report, we evaluated the presence of CuZn-SOD in human serum lipoproteins by both enzyme-linked immunosorbent assay and Western blot analysis of immunoprecipitated lipoprotein samples. The distribution of CuZn-SOD activity among the different lipoprotein fractions was also determined by the xanthine/xanthine oxidase method. The results demonstrated that CuZn-SOD is noticeably present in serum lipoproteins and mainly in low and high density lipoproteins (LDL and HDL). Moreover, experiments performed by incubating CuZn-SOD with a lipid emulsion and subsequent separation of the lipid fraction by ultracentrifugation showed that this enzyme associates in a saturable manner with lipids. The CuZn-SOD bound to LDL and HDL could exert a physiological protective role against oxidative damage of these lipoprotein classes that carry out a crucial role in the cholesterol transport.

Key words: CuZn-superoxide dismutase; Lipoprotein

1. Introduction

Among the antioxidant enzymatic systems, the superoxide dismutase (SOD) isoenzymes are of crucial defense against the reactive oxygen species (ROS) in eukaryotic cells. In mammalians, there are three SODs (EC 1.15.1.1): two intracellular isoenzymes, the mitochondrial Mn-SOD [1] and the cytosolic CuZn-SOD [2], and the extracellular SOD that is considered the major isoenzyme in extracellular space [3]. Among these enzymes which catalyze the dismutation of superoxide to H_2O_2 and O_2, the intracellular enzymes are expressed by all cell types [4,5] whereas the extracellular SOD is less represented [6]. Until now, the only SOD isoenzyme thought to be secreted by fibroblasts and glial cells is the extracellular type; our recent researches, however, showed that human fibroblasts, hepatocarcinoma HepG2 [7], and human neuroblastoma SK-N-BE cells [8] are able to export the cytosolic CuZn-SOD, suggesting an autocrine and/or a paracrine role for this isoenzyme.

The presence of circulating CuZn-SOD, detected by different methods, has so far been explained as deriving from physiological red cells hemolysis. However, the existence of CuZn-SOD secretion not restricted to specific cell lines suggests that serum CuZn-SOD can derive, at least partially, from peripheral tissues as a result of cellular secretion.

The presence of CuZn-SOD in human serum arises the question whether and how this enzyme is transported into the blood. It is known that circulating lipoproteins vehiculate several substances [9]. Among them, there are also antioxidants as vitamins A and E which carry out an important protective effect against lipoprotein oxidation. It is known that low density lipoprotein (LDL) oxidation is an important biochemical event leading to atherosclerosis [10,11]; in fact, oxidized LDL can be detected in atherosclerotic arterial tissue [12] while the resistance of plasma LDL to oxidation is widely assumed to be a good protective indicator against the atherogenetic risk [13].

Here, we investigate whether, like A and E vitamins, the serum CuZn-SOD is carried by circulating lipoproteins.

2. Materials and methods

2.1. Subjects

Ten healthy volunteers, three females and seven males (age 35–50 years), participated in this study giving their informed consent. The subjects were fasted for 12 h before blood collection from a cubital vein puncture.

The serum of each subject was separated after slight centrifugation and 0.1% ethylenediaminetetraacetic acid (EDTA) disodium salt, 0.1% of sodium azide and aprotinin (0.17 TIU/ml) were added in order to prevent the bacterial degradation of proteins.

2.2. Lipoprotein preparation

The lipoprotein collection was performed according to the method of Havel et al. [14]. Very low density (VLDL) and intermediate density lipoproteins (IDL), density 1.006–1.019 g/ml, LDL density 1.019–1.063 g/ml and high density lipoproteins (HDL), density 1.063–1.210 g/ml, were isolated by a tube slicer after centrifugation at 40 000 rpm at 14°C in a 40.3 Beckman rotor for 18, 20 and 44 h for VLDL–IDL, LDL and HDL, respectively.

The lipoprotein fractions and lipoprotein deficient serum (LPDS), representing the bottom fraction obtained after HDL collection [14], were then dialyzed for 24 h against NaCl 0.15 M in the presence of 0.1% EDTA disodium salt and 0.1% of sodium azide at 4°C.

2.3. Enzyme activity assay

The SOD enzymatic activity was carried out by the cytochrome *c*, xanthine/xanthine oxidase method of McCord and Fridovich [2]

*Corresponding author. Fax: (39)-81-7463639.
E-mail: mondola@unina.it

Abbreviations: LDL, low density lipoprotein; VLDL, very low density lipoprotein; HDL, high density lipoprotein; LPDS, lipoprotein deficient serum; EDTA, ethylenediaminetetraacetic acid; SOD, superoxide dismutase; BSA, bovine serum albumin; ANS, 8-anilino-1-naphthalenesulfonic acid magnesium salt

PII: S0014-5793(00)01123-6

modified by Beyer et al. [15] using 50 mM HEPES, 0.1 mM EDTA, pH 7.4, as assay buffer.

2.4. Enzyme-linked immunosorbent assay (ELISA)

ELISA for quantitative detection of human CuZn-SOD was carried out using the Bender Med System kit (Bender Med System Diagnostics GmbH, Vienna, Austria). CuZn-SOD concentrations in serum and in LPDS were determined prediluting the samples 1:100 with phosphate buffer, while the dilution of VLDL was 1:2 and that of LDL and HDL was 1:10. The concentration of CuZn-SOD was determined calculating the average absorbance (450 nm) values for each sample and for the standard curve in duplicate. The detection limit of the assay is 0.07 ng/ml. The intra-assay coefficient of variation is 4%.

2.5. Polarographic method

CuZn-SOD was also determined by a polarographic method [16]. The SOD content is reported as ng of enzyme/ml with reference to the activity in the same conditions of purified sample of human CuZn-SOD. The catalytic constant of this enzyme as measured by polarography was found to be 0.9×10^{9} M^{-1} s^{-1}.

2.6. Immunoprecipitation and Western blot of CuZn-SOD in lipoproteins

The protein concentration of human serum lipoproteins was determined according to the method of Lowry et al. [17] using bovine serum albumin (BSA) as standard. Lipoprotein samples (300 μg) in 1 ml of buffer A (10 ml of Tris–HCl pH 7.4, 150 mM NaCl and 1 mM EDTA) were immunoprecipitated for 1 h at 4°C with rabbit polyclonal anti-human CuZn-SOD (1:100 dilution) prepared as previously described [18]. Protein A Sepharose (CL4B Sigma Chemical Company, St. Louis, MO, USA) was added to samples and continuously stirred for 45 min at 4°C.

After centrifugation in a microfuge at 3000 rpm for 10 min, Sepharose pellets were washed once with 1 ml buffer A plus 1% Triton X-100, 0.4 M NaCl and 0.5% BSA; then the pellets were washed again with the same buffer plus 0.4 M NaCl and then with buffer A alone.

The Sepharose pellets were electrophoresed on a 12% sodium dodecyl sulfate–polyacrylamide gel according to the method of Laemmli [19] together with 25 μg of commercial human erythrocyte CuZn-SOD (Sigma Chemical Company, St. Louis, MO, USA) used as standard. For Western blot experiments, a standard procedure was used utilizing enhanced chemiluminescence detection according to the manifacturer's instructions (Amersham Life Science, UK). The primary rabbit anti-human CuZn-SOD antibodies were the same utilized for the immunoprecipitation experiment at 1:2000 dilution; the secondary antibodies, a horseradish peroxidase-linked donkey anti-rabbit IgG (Amersham Life Science, UK), was instead diluted 1:5000. The detection limit for CuZn-SOD evaluation by Western blot experiments was 5 ng/ml and the intra-assay coefficient of variation was 4.5%.

2.7. Incubation of CuZn-SOD with lipid emulsion

Lipid emulsion (CLINTEC Nutrition Clinique-Montargis, France) obtained by high pressure homogenization was composed of 20% of purified soy oil, 2.5% glycerol, 0.03% sodium oleate, 0.07% phosphatidylethanolamine, 0.01% lyso-phosphatidylethanolamine, 0.9% phosphatidylcholine, 0.022% sphingomyelin, 0.05% lyso-phosphatidylcholine, $<0.02\%$ glycerophosphorylcholine. The emulsion consists of a vesicular dispersion containing a main population of small particles with a mean diameter of 300 nm mixed with very small particles (diameter less than 100 nm) accounting for only 3–6% of total.

75 μl of the lipid emulsion was incubated with different amounts of human CuZn-SOD dissolved in 6 ml of NaCl 0.15 M.

The lipid–CuZn-SOD mixture, incubated at room temperature for 30 min, was centrifuged at 30 000 rpm for 45 min in a 40.3 Beckman rotor. The top layer and the bottom fractions were separated by tube slicer and the activity of CuZn-SOD was evaluated by the cytochrome *c*, xanthine/xanthine oxidase method.

2.8. Fluorimetric titration of a fluorescent probe with lipid emulsion

To evaluate whether the lipid emulsion was organized as a mixed micelles association or as a vesicular dispersion, we utilized a fluorimetric method [20] that uses 8-anilino-1-naphthalenesulfonic acid magnesium salt (ANS) as fluorescent probe. Fluorimetric titration was performed by adding increasing amounts of lipid emulsion to a saline solution (0.15 M NaCl) containing 10 μM ANS. The excitation and emission wavelengths were 375 and 520 nm, respectively.

3. Results

The CuZn-SOD distribution between serum and LPDS and among the different lipoprotein classes was evaluated using different methodologies.

Fig. 1A shows the CuZn-SOD serum concentration determined by an ELISA assay. The total serum enzyme concentration is 115 ± 20 ng/ml (mean ± S.E.M., $n=10$); $45\pm3\%$ of CuZn-SOD is detectable in LPDS and the remaining part in lipoproteins. Assuming as 100% the amount of CuZn-SOD associated with lipoproteins, $50\pm6\%$ (mean ± S.E.M., $n=10$) of the enzyme is detected in LDL, $36\pm2\%$ in HDL and the remaining part in VLDL–IDL (Fig. 1B). Further evidence that CuZn-SOD is bound to serum lipoproteins is obtained by immunoprecipitation experiments in which CuZn-SOD is detected in all lipoprotein classes, even if a precise quantification from these data cannot be performed (Fig. 1C).

We also measured CuZn-SOD activity by the cytochrome *c*/xanthine/xanthine oxidase method. Since this assay does not allow to detect low CuZn-SOD activity values, in order to evaluate the distribution of enzyme activity in human serum

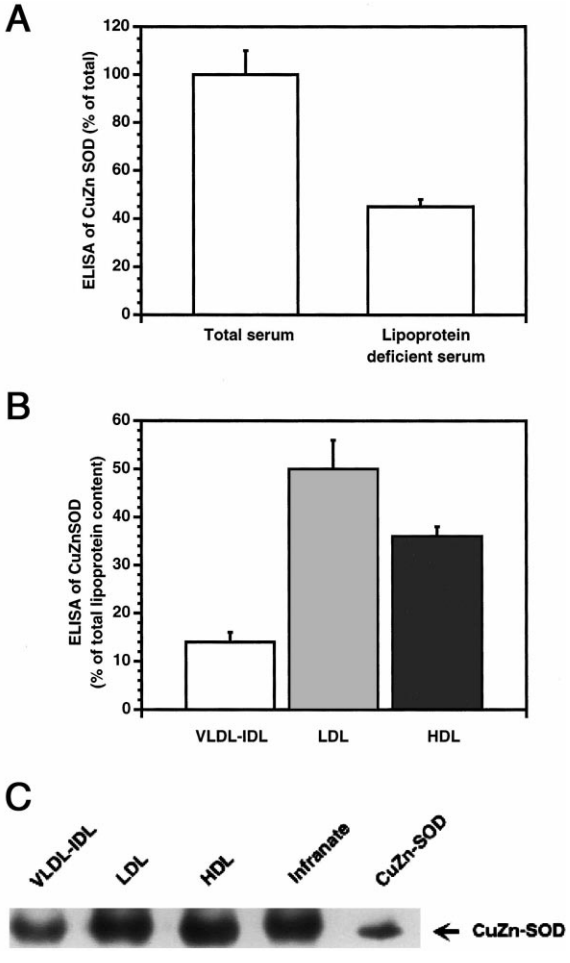

Fig. 1. CuZn-SOD protein concentration determined by the ELISA method in total and in LPDS (A). Values are expressed as percent of total serum CuZn-SOD concentration (115 ± 20 ng/ml). In (B), the CuZn-SOD distribution in lipoprotein fractions is expressed as percent values assuming as 100% the amount of enzyme detected in lipoproteins. The values are means ± S.E.M. ($n=10$). In (C), the Western blot of CuZn-SOD present in lipoproteins precipitated with a rabbit polyclonal anti-human SOD is illustrated.

lipoproteins, we incubated 4.5 ml of pooled human serum with 200 U of this enzyme at 37°C for 30 min, before starting for lipoproteins isolation. The activity of CuZn-SOD in LPDS is about 40 ± 3% (mean ± S.E.M., $n = 3$) of total serum CuZn-SOD (Fig. 2A), showing that the main CuZn-SOD activity is present in lipoproteins. Among lipoprotein classes, we have observed the presence of CuZn-SOD activity mainly in LDL (46 ± 5% of total lipoprotein content, mean ± S.E.M., $n = 3$). The amount present in HDL and VLDL–IDL was 31 ± 5% and 23 ± 4%, respectively (Fig. 2B). The CuZn-SOD concentration in the same samples was also determined by ELISA assay. The enzyme distribution in LPDS and lipoproteins was 45 ± 4% and 55 ± 6% (means ± S.E.M., $n = 3$), respectively. Assuming as 100% the amount of CuZn-SOD associated with lipoproteins, the percent values detected in lipoproteins were 20 ± 5, 43 ± 8 and 37 ± 6 (means ± S.E.M., $n = 3$) for VLDL–IDL, LDL and HDL, respectively.

These results are further confirmed by the polarographic method in which the enzyme activity in lipoprotein compared to that recovered in LPDS shows a very similar distribution to that obtained by either cytochrome *c*/xanthine/xanthine oxidase and ELISA assays (45 ± 3% in LPDS; 55 ± 5% in serum lipoproteins, means ± S.E.M., $n = 10$).

In order to evaluate whether CuZn-SOD has the ability to bind the lipid component of lipoproteins, different amounts of CuZn-SOD were incubated with lipid emulsion and further ultracentrifuged to separate the lipids from the aqueous phase (Fig. 3). At CuZn-SOD concentrations lower than 20 U, all the enzyme added to lipid emulsion was detected in the top lipid phase. As the CuZn-SOD concentration increases over 20 U, the enzyme activity is detected also in the bottom fraction.

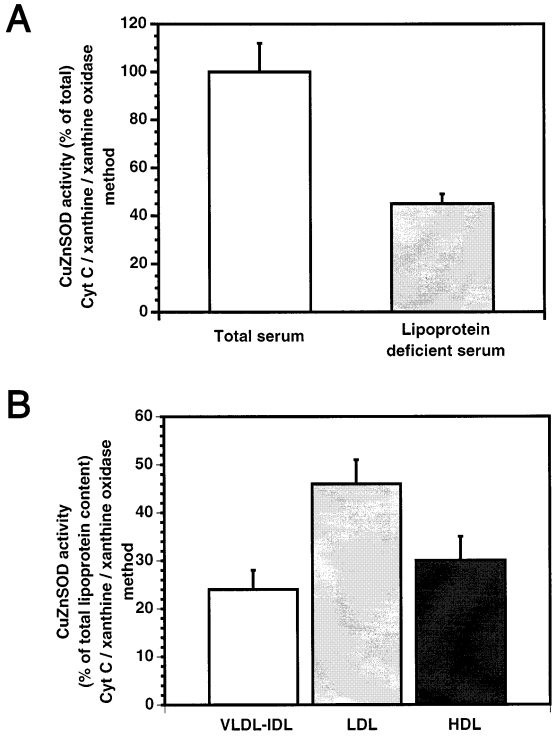

Fig. 2. CuZn-SOD activity determined in total and in LPDS (A). Values are expressed as percent of total serum CuZn-SOD activity. In (B), the CuZn-SOD activity distribution in lipoprotein fractions is expressed as percent values assuming as 100% the amount of enzyme activity detected in lipoproteins. These values (means ± S.E.M., $n = 3$) were obtained incubating 4.5 ml of serum with 200 U of CuZn-SOD at 37°C for 30 min before starting the lipoprotein collection.

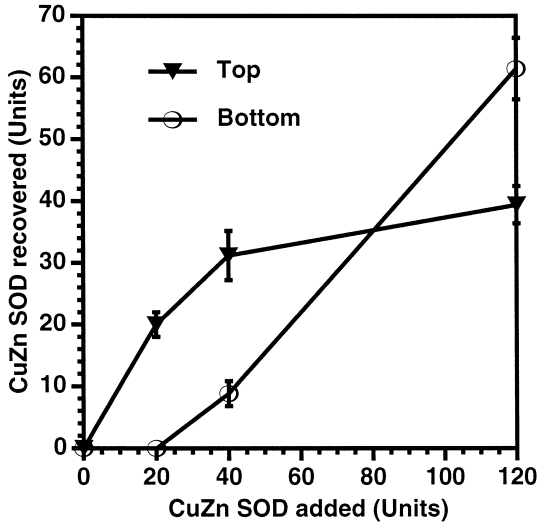

Fig. 3. CuZn-SOD activity in the top and bottom fractions (infranate) obtained by ultracentrifugation of a lipid emulsion preincubated with an increasing amount of enzyme. SOD activity was measured by the cytochrome *c*/xanthine/xanthine oxidase method. Values are means ± S.E.M. of three experiments performed in duplicate.

To evaluate the physical form of lipid emulsion association, we performed a fluorimetric titration of the fluorescent probe ANS with increasing lipid emulsion concentrations ranging between 3×10^{-4}% and 5%; the higher concentration used corresponds to that reached at the top layer obtained after ultracentrifugation of the CuZn-SOD–lipid emulsion mixture. The data obtained do not show the sharp increase of fluorescence emission characteristic of the phase transition corresponding to mixed micelle formation; these data demonstrate that the lipid emulsion interacts with CuZn-SOD in the form of vesicular dispersion (data not shown). Consequently, we strongly assume that CuZn-SOD is bound with the lipid emulsion by hydrophobic interaction.

4. Discussion

In this study, we show, for the first time, that serum CuZn-SOD is bound to circulating lipoproteins. The analysis of CuZn-SOD distribution between the different lipoprotein classes shows that this enzyme is mainly bound to LDL and HDL (density included between 1.019 and 1.210 g/ml) since the CuZn-SOD amount present in VLDL–IDL was about 20% of the total CuZn-SOD lipoprotein content.

This finding has relevant physiological implications. In fact, ROS mediating lipid peroxidation of LDL are considered to have a key role in the pathogenesis of atherosclerosis. Oxidized LDLs have an increased half life and are preferentially recognized by scavenger receptors which, unlike the B/E receptor, are not subjected to down-regulation [21], leading to foam cells formation. It is known that LDL represents the principal form of transport of vitamin A and E [22,23] and the presence of these antioxidants has been positively correlated with increased resistance to the oxidative modification of

LDL. An enhanced resistance to oxidation of LDL and a decreased lipid peroxidation have been shown to occur during β carotene supplementation to patients affected by cystic fibrosis in which a severe β carotene deficiency is demonstrated [24]. The presence of CuZn-SOD in circulating LDL could carry out, like vitamins A and E, an important role limiting the oxidative modification of this lipoprotein; in particular, it could exert a specific role protecting these particles from superoxide-mediated lipid peroxidation [25,26]. There are, in fact, several evidences of $O_2^{-\bullet}$ involvement in the LDL oxidation mechanism: (i) endothelial cells have been demonstrated to produce both nitric oxide and superoxide that represent potent LDL oxidants [27]; (ii) cultured smooth cells and endothelial cells stimulate $O_2^{-\bullet}$-mediated LDL oxidation in the presence of transitional metals that are reduced by $O_2^{-\bullet}$ [28]; (iii) the addition of CuZn-SOD in vitro inhibits monocyte-mediated LDL oxidation [29].

Some recent data showed that the oxidative susceptibility of LDL in vitro and in vivo is influenced by HDL. This lipoprotein, which is involved in the reverse cholesterol transport [30], has been reported to exhibit an antioxidant effect since oxidative modification occurs more slowly in LDL–HDL mixtures than in LDL alone [9,31–34]. Using the oxidation-labile fluorescent probe parinaric acid cholesteryl ester, direct evidence has been given that HDL inhibits both Cu^{+}- and Fe^{2+}-induced peroxidation of LDL-associated lipids [35]. The presence of transition metals is fundamental for in vitro LDL oxidation as demonstrated by the inhibition of lipoprotein peroxidation by metal chelators [29]. A possible mechanism for the antioxidant effects of HDL has been related to inhibition of transitional metal catalyzing reactions due to the presence of transferrin and ceruloplasmin in apo A_I containing lipoproteins [9]. The presence of CuZn-SOD in HDL can, at least partially, account for the antioxidant effect of this lipoprotein class.

Finally, in addition to the presence of CuZn-SOD in circulating lipoproteins, we clearly demonstrated that CuZn-SOD interacts in a saturable manner with emulsified lipids; when 20 U of CuZn-SOD was added to the lipid emulsion, all the enzyme activity was recovered in the lipid top layer separated from the aqueous phase by ultracentrifugation. Since the lipid emulsion used for such experiments is organized as vesicular dispersion, we can assume that CuZn-SOD associates with lipids by a simple hydrophobic interaction. These data suggest that CuZn-SOD is bound to the lipid component of lipoproteins. However, further studies could better clarify the association of CuZn-SOD with specific lipid components of lipoproteins and evaluate the existence of an interaction with the apoprotein component of lipoproteins.

Acknowledgements: The authors are grateful to Prof. Giuseppe Rotilio for critical revision of the manuscript and Prof. Giuseppe Palumbo for his valuable comments and suggestions. The authors also thank Mr. Lucio Cammarota for his technical assistance. This work was supported in part by the Associazione Italiana Ricerca sul Cancro (A.I.R.C.).

References

[1] Weisiger, R.A. and Fridovich, I. (1973) J. Biol. Chem. 248, 4793–4796.
[2] McCord, J.M. and Fridovich, I. (1969) J. Biol. Chem. 244, 6049–6055.
[3] Marklund, S.L. and Bjelle, A. (1986) Ann. Rheum. Dis. 45, 847–851.
[4] Marklund, S.L., Westman, G., Lundgren, E. and Roos, G. (1982) Cancer Res. 42, 1955–1961.
[5] Marklund, S.L. (1990) Biochem. J. 266, 213–219.
[6] Marklund, S.L. (1984) Biochem. J. 222, 649–655.
[7] Mondola, P., Annella, T., Santillo, M. and Santangelo, F. (1996) Int. J. Biochem. Cell Biol. 28, 677–681.
[8] Mondola, P., Annella, T., Serù, R., Santangelo, F., Iossa, S., Gioielli, A. and Santillo, M. (1998) Brain Res. Bull. 45, 517–520.
[9] Kunitake, S.T., Jarvis, M.R., Hamilton, R.L. and Kane, J.P. (1992) Proc. Natl. Acad. Sci. USA 89, 6993–6997.
[10] Steinberg, D., Parthasarathy, T.E., Carew, J.C., Khoo and Witztum, J.L. (1989) N. Engl. J. Med. 320, 915–924.
[11] Heinecke, J.W. (1997) Curr. Opin. Lipidol. 8, 268–274.
[12] Yla-Herttuala, S., Palinski, W., Rosenfeld, M.E., Parthasarathy, S., Carew, T.E., Butler, S., Witztum, J.L. and Steinberg, D. (1989) J. Clin. Invest. 84, 1086–1095.
[13] O'Leary, V.L., Tilling, G., Fleetwood, D., Stone, D. and Darley-Usmar, V. (1966) Atherosclerosis 119, 169–179.
[14] Havel, R.S., Eder, H.A. and Bragdon, S.H. (1955) J. Clin. Invest. 34, 1345–1353.
[15] Beyer Jr., W.F., Wang, Y. and Fridovich, I. (1986) Biochemistry 25, 6084–6088.
[16] Rigo, A., Viglino, P. and Rotilio, G. (1975) Anal. Biochem. 68, 1–8.
[17] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[18] Mondola, P. (1993) Comp. Biochem. Physiol. 105B, 457–464.
[19] Laemmli, U.K. (1970) Nature 227, 680–685.
[20] De Vendittis, E., Palumbo, G., Parlato, G. and Bocchini, V. (1981) Anal. Biochem. 115, 278–286.
[21] Brown, M.S. and Goldstein, J.L. (1983) Annu. Rev. Biochem. 52, 223–261.
[22] Krinsky, N.I., Cornwell, D.G. and Oncley, J.L. (1958) Arch. Biochem. Biophys. 73, 233–236.
[23] Esterbauer, H., Dieber-Rotheneder, M., Striegl, G. and Waeg, G. (1991) Am. J. Clin. Nutr. 53, 314S–321S.
[24] Winklhofer-Roob, B.M., Puhl, H., Khoschsorur, G., van't Hof, M.A., Esterbauer, H. and Shmerling, D.H. (1995) Free Radic. Biol. Med. 5, 849–859.
[25] Sato, K., Niki, E. and Shimasaki, H. (1990) Arch. Biochem. Biophys. 279, 402–405.
[26] Stocker, R., Bowry, V.W. and Frei, B. (1991) Proc. Natl. Acad. Sci. USA 88, 1646–1650.
[27] Darley-Usmar, V.M., Hogg, N., O'Leary, V.J., Wilson, M.T. and Moncada, S. (1992) Free Radic. Res. Comm. 17, 9–20.
[28] Mukhopadhyay, C.K., Ehrenwald, E. and Fox, P.L. (1996) J. Biol. Chem. 271, 14773–14778.
[29] Xing, X., Baffic, J. and Sparrow, C.P. (1998) J. Lipid Res. 39, 2201–2208.
[30] Johnson, W.J., Mahlberg, G.H., Rothblat, J.H. and Phillips, M.C. (1991) Biochim. Biophys. Acta 1085, 273–298.
[31] Parthasarathy, S., Barnett, S. and Fong, L.G. (1990) Biochim. Biophys. Acta 1044, 275–283.
[32] Ohta, T., Takata, K., Horiuchi, S., Morino, Y. and Matsuda, I. (1989) FEBS Lett. 257, 435–438.
[33] Mackness, M.I., Arrol, S., Abbott, C. and Durrington, P.N. (1993) Atherosclerosis 104, 129–135.
[34] Mackness, M.I., Abbott, C., Arrol, S. and Durrington, P.N. (1993) Biochem. J. 294, 829–834.
[35] Tribble, D.L., Chu, B.M., Gong, E.L., van Venrooij, F. and Nichols, A.V. (1995) J. Lipid. Res. 36, 2580–2589.