Oxidized low-density lipoprotein induces the production of superoxide by neutrophils

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Abstract Exposure of guinea pig peritoneal neutrophils to ox-LDL led to the production of superoxide, which was measured by the formation of superoxide-dependent chemiluminescence. The cells exposed to unoxidized LDL, e.g. native LDL, acetyl-LDL, and self-aggregates of LDL showed no production of superoxide. The superoxide production was correlated with the levels of oxidative modification of LDL and reached a maximum between 10 and 30 min during incubation, but preincubating the cells with cytochalasin B decreased the superoxide production. These findings indicate that neutrophils rapidly take up ox-LDL by phagocytosis and generate superoxide which may cause superoxide-mediated lipid peroxidation in vivo.

Key words: Neutrophil; Oxidized LDL; Superoxide; Chemiluminescence; Lipid peroxidation; Atherosclerosis

1. Introduction

The oxidative modification of low-density lipoprotein (LDL) has been implicated in the pathogenesis of atherosclerosis [1,2]. The oxidized LDL (ox-LDL) is rapidly taken up by macrophages through its scavenger receptors and leads to the formation of lipid-laden macrophages (or foam cells) in the early stages of atherosclerosis [2]. During the oxidation of LDL, the polyunsaturated fatty acids in the LDL lipids are, in part, converted to lipid hydroperoxides and then to some aldehydes, which can alter the protein moiety of LDL (or apolipoprotein B) [3,4]. The aldehydes formed in the process of lipid peroxidation of LDL are known to give fluorescent lipid peroxidation products, presumably resulting from the reaction of aldehydes with the free amino groups (ε-NH_2) of lysyl residues in protein [3,5]. The fluorescent ox-LDL is characterized by higher anionic electrophoretic mobility due to the blockage of lysyl residues [6], and is known as a strong atherogenic form [7,8]. There is increasing evidence that the oxidative modification of LDL contributes to the formation of form cells in the artery wall [9], but the mechanisms of in vivo lipid peroxidation of LDL remain unknown.

Neutrophils, phagocytic cells of the immune system, have been shown to produce superoxide anion radicals (O_2^-) [10,11]. The chemical reactivity of superoxide may be limited [12], but, in the presence of transition metal ions, the partially reduced forms of superoxide such as hydrogen peroxide (H_2O_2) and the hydroxyl radical ($HO^\bullet$) are possibly initiators of lipid peroxidation in the physiological condition [13,14]. Moreover, a variety of cell types in the artery wall, including macrophages, have been shown to produce nitric oxide ($NO^\bullet$), which reacts with superoxide and yields potent prooxidants (e.g. peroxynitrite anion (^-OONO)), by oxidizing arginine with a NADPH-dependent enzyme [15]. The prooxidant effect of $NO^\bullet$ in the presence of superoxide has been shown to modify LDL to an oxidized and atherogenic form [16,17].

In the present study, we have investigated the interaction of ox-LDL with neutrophils, which are capable of generating superoxide anion radicals. The data show that neutrophils recognize oxidatively modified LDL and lead to the superoxide production which is correlated with the level of fluorescence intensity of ox-LDL as well as its relative electrophoretic mobility.

2. Experimental

2.1. Materials

2-Methyl-6-phenyl-3,7-dihydroimidazo(1,2-a)pyrazin-3-one (CLA) was purchased from Tokyo Kasei Co. (Tokyo, Japan). Phorbol-12-myristate-13-acetate (PMA), xanthine oxidase, hypoxanthine, superoxide dismutase (SOD), glycogen, zymosan, cytochalasin B, and copper (II) sulfate were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Opsonized zymosan was prepared by the method of Johnston et al. [18]. Human low-density lipoprotein (LDL) was isolated from the plasma of individual healthy males, by ultracentrifugation (d. 1.019–1.063 g/ml) [19]. Acetyl-LDL was prepared by the method of Basu et al. [20]. LDL self-aggregates were prepared from native LDL by vortexing for 1 min as described by Khoo et al. [21].

2.2. Preparation of neutrophils

Neutrophils were obtained from the peritoneal cavities of guinea pigs (Hartley, 280–300 g/B.W. female) that had been injected intraperitoneally with 20 ml of saline solution containing 0.4 g glycogen 16 h previously. After removing erythrocytes by hypotonic lysis at 4°C, neutrophils pelleted by centrifugation were resuspended in Hank's balanced salt solution without Phenol red (NBSS, Nissui Pharmaceutical Co., Tokyo) at a cell density of 2×10^7 cells/ml. The neutrophils (>95%) were immediately used for experiments.

2.3. LDL oxidation and characterization

Samples of native LDL were diluted to 2 mg protein/ml with phosphate-buffered saline (PBS, 10 mM, pH 7.4) and incubated at 37°C in the presence of 25 μM $CuSO_4$ for up to 24 h [19]. The development of fluorescent products was measured as described by Shimasaki [22] with a slight modification. A 0.1 ml aliquot of modified-LDL suspension (2 mg protein/ml) was diluted with 5 ml of PBS, and the intensity of fluorescence was measured at a fluorescence maximum of 430 nm and an excitation maximum of 365 nm with a fluorescence spectrophotometer (204, Hitachi Co., Japan). The fluorescence intensity was expressed as the molar ratio relative to those of quinine sulfate (0.1 μM in 0.1 N H_2SO_4) measured at a fluorescence maximum of 448 nm and an excitation maximum of 352 nm. The relative electrophoretic mobility (REM) was determined by performing agarose gel electrophoresis as described by Noguchi et al. [4]. Oxidation of LDL was also monitored spectrophotometrically by measuring the formation of conjugated diene (233 nm) and the level of thiobarbituric acid-reactive substances (TBA-RS, 532 nm) [23].

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2.4. Chemiluminescence measurement

The production of superoxide by neutrophils was measured by the formation of superoxide-dependent chemiluminescence [24], which was monitored with a luminescence counter (Lumi Counter 1000, Nichi-On Medical and Physical Instruments Co., Japan). To measure chemiluminescence, 600 μl of a mixture which consisted of 50 μl of neutrophils (1×10^6 cells) and 200 μl of ox-LDL (final conc. 0.65–0.68 protein mg/ml) in HBSS (pH 6.0) was placed in the reaction chamber. The mixture was preincubated with gentle mixing for 2 min at 37°C, and then 100 μl of CLA (6 μM in HBSS) was injected into the mixture with a microliter syringe. The amount of chemiluminescence was determined as the maximal light intensity with an integrator-timer (SS-100F recorder, Nichi-On Medical and Physical Instruments Co.).

3. Results

Guinea pig peritoneal neutrophils exposed to ox-LDL led to the production of superoxide, which was measured by the formation of superoxide-dependent chemiluminescence in the presence of CLA. The chemiluminescence was completely inhibited by the addition of SOD (final conc. 5 μg/ml) under the same conditions. There was a good relationship between the production of superoxide by the cells and the levels of oxidative modification of LDL. Fig. 1A shows the superoxide-dependent chemiluminescence (or superoxide production) which is increased linearly with the fluorescence intensity of ox-LDL. The cells with native LDL were not stimulated under the same conditions, and the superoxide-dependent chemiluminescence was found to be at the control level.

The levels of oxidation of LDL were also monitored by agarose gel electrophoresis (Fig. 1B). The relative electrophoretic mobility (REM), which was determined as the ratio of the migrating distance of ox-LDL to that of native LDL, was correlated with the development of the superoxide-dependent chemiluminescence. The generation of both conjugated diene and TBARS observed in the ox-LDL was not related to the chemiluminescence. A major part of TBARS (>90%) in the ox-LDL suspension was water soluble materials which were lost after dialysis against phosphate-buffered saline (PBS). It should be noted that neutrophils with acetyl-LDL as well as LDL self-aggregates, freshly prepared from native LDL by vortexing, were not stimulated and had no superoxide-dependent chemiluminescence under the same conditions (data not shown).

The activation of neutrophils by ox-LDL was compared with that by two other stimulants, PMA and opsonized zymosan, under the same conditions. The production of superoxide by the PMA-stimulated cells was increased with the concentrations of PMA between 0 and 100 ng/ml, and reached its maximum and a plateau thereafter (Fig. 2A). In contrast, the production of superoxide by the cells treated with ox-LDL as well as opsonized zymosan reached its maximum, and then subsequently fell in a higher concentration of the stimulants (Fig. 2B,C).

The time course of superoxide production by the ox-LDL-treated cells was similar to that of superoxide production by the opsonized zymosan-treated cells, and both of these reached maximum superoxide production between 10 and 30 min, before falling slowly to resting levels (Fig. 3). On the other hand, the superoxide production by neutrophils with PMA rapidly decreased during incubation of the cells. Preincubation of neutrophils with cytochalasin B decreased the superoxide production, when the cells were stimulated with ox-LDL. In contrast, PMA-induced superoxide production as well as zymosan-induced superoxide production increased slightly after preincubation of the cells with cytochalasin B (Fig. 4).

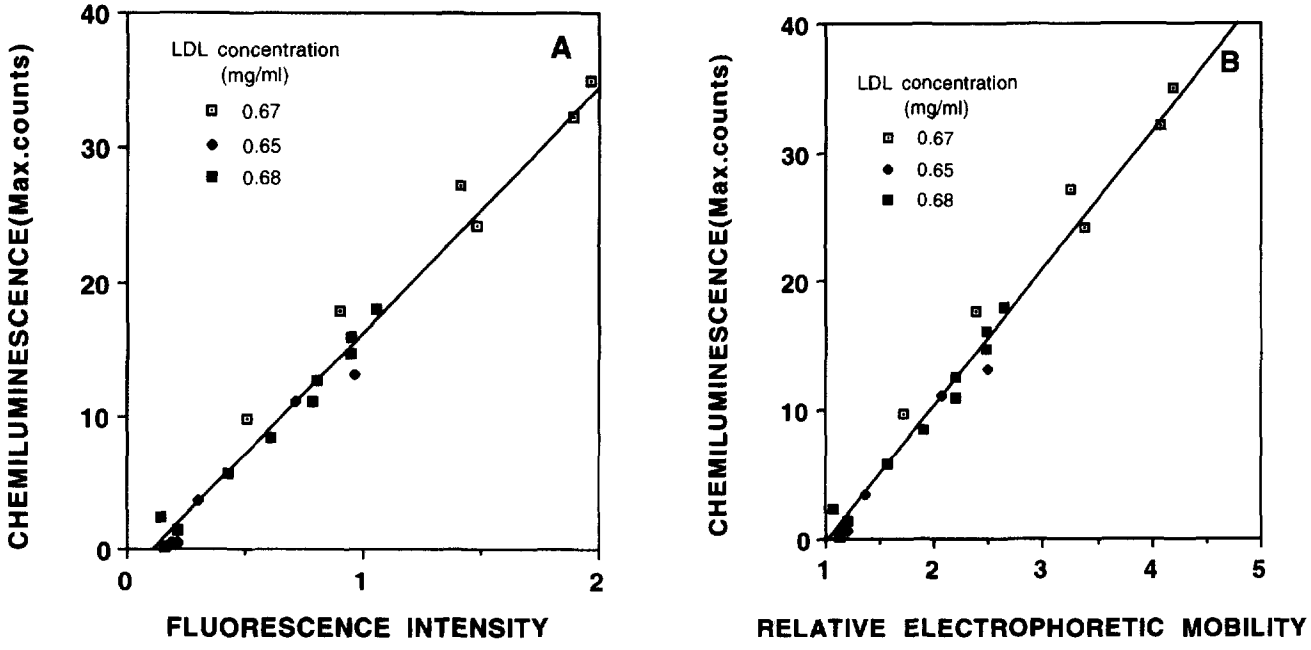

Fig. 1. Relationship between superoxide-dependent chemiluminescence (maximum counts) and (A) relative fluorescence intensity, and (B) relative electrophoretic mobility (REM) of ox-LDL. The reaction mixture (600 μl, HBSS pH 6.0) contained CLA (1 μM), guinea pig peritoneal neutrophils (1×10^6 cells), and ox-LDL (0.65–0.68 mg protein/ml) obtained from the different oxidation levels of LDL. (A) The fluorescence intensity of ox-LDL was measured as described in section 2. (B) Agarose gel electrophoresis was carried out in barbital buffer solution, pH 8.8. The relative electrophoretic mobility (REM) of ox-LDL was determined as the ratio of the migrating distance of ox-LDL to that of native-LDL at zero time.

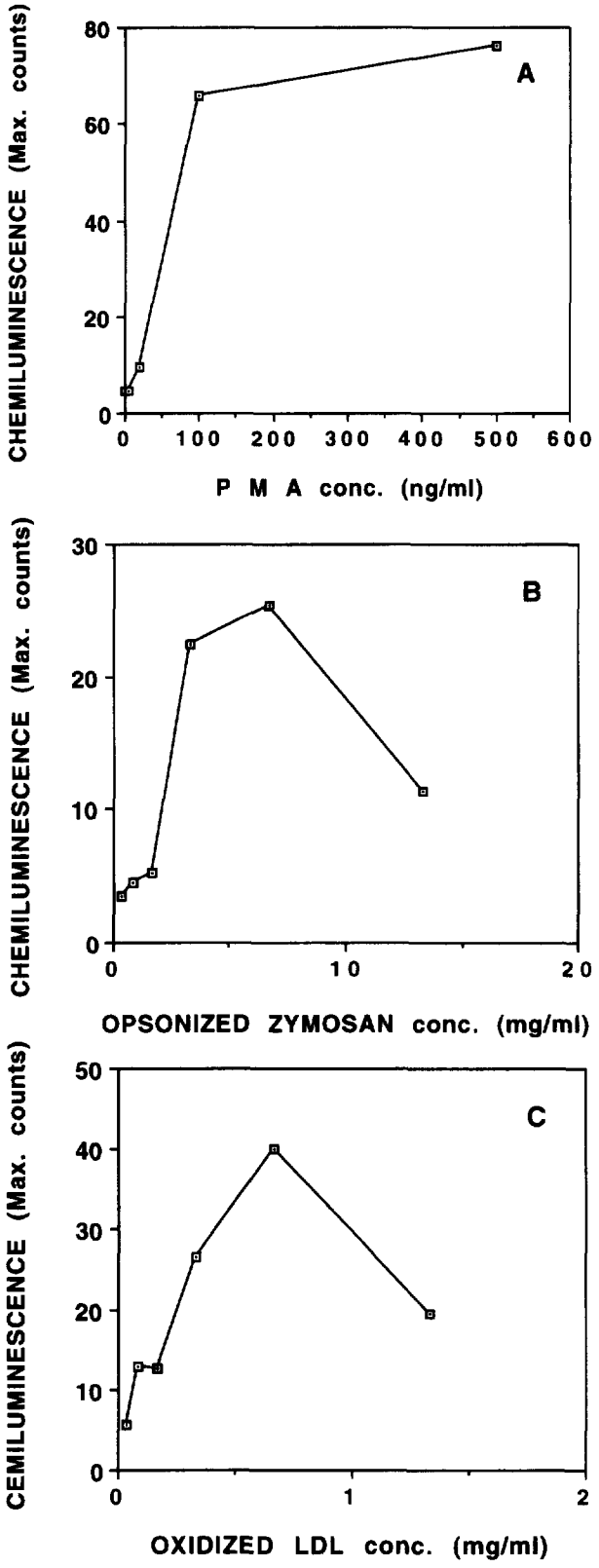

Fig. 2. Effects of the concentration of the stimulants PMA, opsonized zymosan and ox-LDL on the production of superoxide by guinea pig peritoneal neutrophils. The incubation mixtures (600 μl, HBSS pH 6.0) contained CLA (1 μM), neutrophils (1×10^6 cells), and one of the following stimulants. (A) PMA (0–500 ng/ml), (B) opsonized zymosan (0.33–13.3 mg/ml) or (C) ox-LDL (0.03–1.3 mg protein/ml) obtained from ox-LDL with a fluorescence intensity of 1.41 (see Fig. 1).

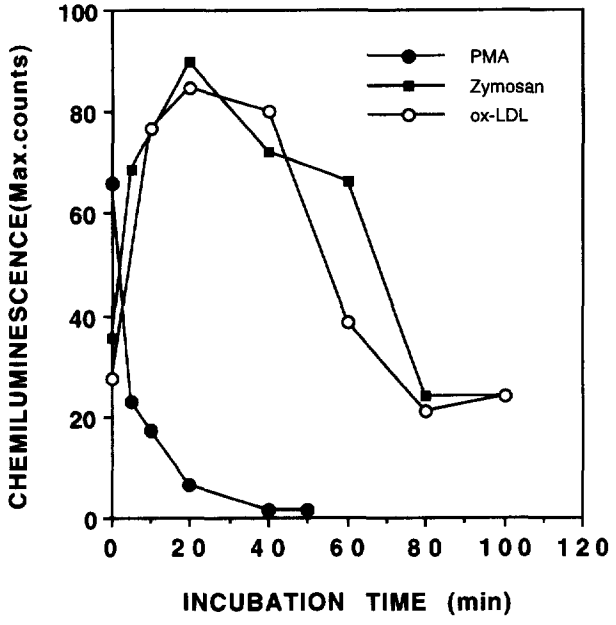

Fig. 3. Time course of superoxide production by guinea pig peritoneal neutrophils treated with PMA, opsonized zymosan, or ox-LDL. The incubation mixtures (600 μl, HBSS pH 6.0) contained CLA (1 μM), neutrophils (1×10^6 cells), and one of the following stimulants, PMA (100 ng/ml), opsonized zymosan (3 mg/ml) or ox-LDL (0.65 mg protein/ml) obtained from ox-LDL with the fluorescence intensity of 1.04 (see Fig. 1).

4. Discussion

The exposure of guinea pig peritoneal neutrophils to ox-LDL led to the production of superoxide which correlated with the levels of the oxidative modification of LDL monitored with a relative fluorescence intensity and a relative electrophoretic mobility. In contrast, neutrophils exposed to unoxidized LDL, such as native LDL, acetyl-LDL, and self-aggregates of LDL exhibit no production of superoxide under the same conditions. It should be noted that ox-LDL without neutrophils generates a short-lived chemiluminescence peak immediately after the addition of CLA, but the production of chemiluminescence by ox-LDL differs from the superoxide production by the cells treated with ox-LDL (data not shown).

The production of superoxide by ox-LDL-treated cells increased progressively with incubation time for up to 20 min at 37°C, and the time course of the superoxide production was similar to that for opsonized zymosan-treated cells. The examination of ox-LDL by electron microscopy and gel permeation by HPLC demonstrated the formation of ox-LDL aggregates as described previously [19]. We therefore considered the possibility that the ox-LDL aggregates are taken up by phagocytosis. Cytochalasin B, which is known to disrupt the contractile microfilament system and causes retraction of the functions of phagocytic cells [25], inhibited, at least in part, the production of superoxide by the ox-LDL-treated cells, suggesting that uptake occurred predominantly due to phagocytosis. The mechanism of superoxide production by ox-LDL-treated cells is not yet known, but it differs from the mechanisms of superoxide production by the PMA-treated and opsonized zymosan-treated cells which were not inhibited by cytochalasin B.

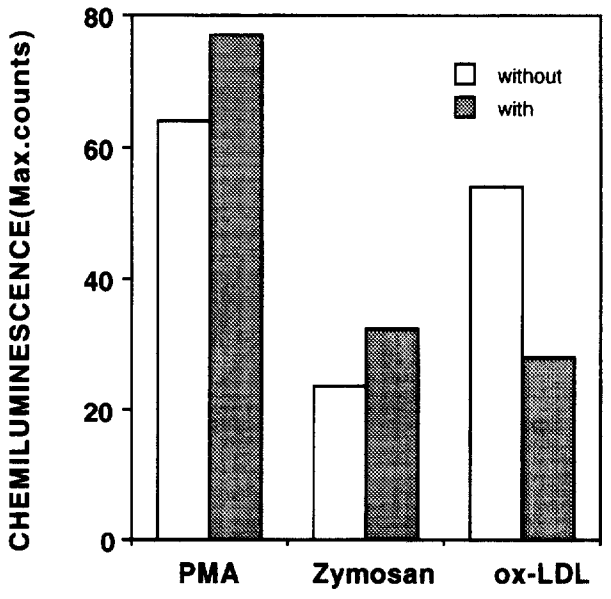

Fig. 4. Effect of cytochalasin B on the production of superoxide by guinea pig peritoneal neutrophils treated with PMA, opsonized zymosan, and ox-LDL. Neutrophils were incubated for 2 min with one of the following stimulants, PMA, opsonized zymosan or ox-LDL, with and without the addition of 5 μl cytochalasin B (2 mg/ml, DMSO). Other conditions were as given in the legend to Fig. 3.

It has become evident that the oxidative modification of LDL which occurs in vivo may play an important role in the early stages of atherogenic disease [2,26]. Although macrophages are unable to efficiently accumulate native LDL, it is believed that ox-LDL is able to recruit monocytes and induce lipid-laden macrophages, termed foam cells, in atherosclerotic lesions, but the in vivo mechanism of LDL oxidation remains unclear. In particular, a large amount of ox-LDL may be required by the macrophages in atherosclerotic lesions.

The neutrophils produce superoxide anion radicals [10,11], which may be important in the in vivo mechanism of LDL oxidation. It has been suggested that the partially reduced forms of superoxide, e.g. hydrogen peroxide (H_2O_2), which freely passes through membranes and can reach any cellular compartment, and the hydroxyl radical ($HO^{\bullet}$), which is produced from H_2O_2 in the presence of intracellular iron or copper ions via Fenton reaction, may initiate in vivo lipid peroxidation [13,14]. Moreover, it was recently reported that macrophages as well as many of the cell types associated with atherosclerotic lesions produce $NO^{\bullet}$ which rapidly reacts with superoxide to form peroxynitrite anion ($^{-}OONO$) [15], and that the modification of LDL caused by the prooxidant leads to an oxidized and potentially atherogenic form [16,17]. Preliminary experiments in this laboratory showed that the production of superoxide by neutrophils is much greater than that by macrophages when these cells are incubated with ox-LDL. Hence a possible explanation for the in vivo mechanism of LDL oxidation is that a trace amount of ox-LDL may stimulate neutrophils as well as macrophages, resulting in the production of superoxide, which may produce the partially reduced forms of superoxide in the presence of transition metal ions, or may produce the peroxynitrite anion in the presence of $NO^{\bullet}$, initiating lipid peroxidation in a very early stage of the injury. Although the lipid peroxidation of LDL may be developed with an autocatalytic reaction if ox-LDL is present, the production of superoxide by ox-LDL-stimulated neutrophils may be a major source of the initiators of the in vivo lipid peroxidation which results in the oxidative modification of native LDL that accumulates lipids during foam cell formation.

References

[1] Steinberg, D., Parthasarathy, S., Carew, T.E., Khoo, J.C. and Witztum, J.L. (1989) N. Engl. J. Med. 320, 915–924.
[2] Geng, Y.J. and Hansson, G.K. (1992) J. Clin. Invest. 89, 1322–1330.
[3] Singh, S., Suri, R. and Agrawal, C.G. (1995) Biochim. Biophys. Acta 1254, 135–139.
[4] Noguchi, N., Gotoh, N. and Niki, E. (1994) Biochim. Biophys. Acta 1213, 176–182.
[5] Jürgens, G., Lang, J. and Esterbauer, H. (1986) Biochim. Biophys. Acta 875, 103–114.
[6] Cominacini, L., Garbin, U., Davoli, A., Micciolo, R., Bosello, O., Gaviraghi, G., Scuro, L.A. and Pastorino, A.M. (1991) J. Lipid Res. 32, 349–358.
[7] Fogleman, A.M., Schechter, T., Seager, J., Hokom, T., Child, J.S. and Edwards, P.A. (1980) Proc. Natl. Acad. Sci. USA 77, 2214–2219.
[8] Quinn, M.T., Parthasarthy, S., Fong, L.G. and Steinberg, D. (1987) Proc. Natl. Acad. Sci. USA 84, 2995–2998.
[9] Kita, T., Nagano, Y., Yokoda, M., Ishii, K., Kume, N., Ooshima, A., Yoshida, H. and Kawai, C. (1987) Proc. Natl. Acad. Sci. USA 84, 5928–5931.
[10] Edwards, S.W. and Lloyd, D. (1988) FEBS Lett. 226, 39–42.
[11] Rist, R. and Naftalin, R.J. (1993) Biochem. J. 291, 509–514.
[12] Fridovich, I. and Freeman, B.A. (1986) Annu. Rev. Physiol. 48, 693–702.
[13] Halliwell, B. (1981) in: Age Pigments (Sohal, R.S., Ed.) pp. 1–62.
[14] Halliwell, B. and Gutterige, J.M.C. (1989) in: Free Radicals in Biology and Medicine, 2nd Edn., Clarendon Press, Oxford.
[15] Radi, R., Beckman, J.S., Bush, K.M. and Freeman, B.A. (1991) J. Biol. Chem. 266, 4244–4250.
[16] Darley-Usmar, V.M., Hogg, N., O'Leary, V.J., Wilson, M.T. and Moncada, S. (1992) Free Rad. Res. Commun. 17, 9–20.
[17] Hogg, N., Darley-Usmar, V.M., Graham, A. and Moncada, S. (1993) Biochem. Soc. Trans. 10, 358–362.
[18] Johnston, R.B. Jr., Keel, B.B. Jr., Misra, H.P., Lehmeyer, J.E., Webb, L.S., Baemer, R.L. and Rajagopalan, K.V. (1975) J. Clin. Invest. 55, 1357–1372.
[19] Maeba, R., Shimasaki, H. and Ueta, N. (1994) Biochim. Biophys. Acta 1215, 79–86.
[20] Basu, S.K., Goldstein, J.L., Anderson, R.G.W. and Brown, M.S. (1976) Proc. Natl. Acad. Sci. USA 73, 3178–3182.
[21] Khoo, J.C., Miller, E., McLoughlin, P. and Steinberg, D. (1988) Arteriosclerosis 8, 348–358.
[22] Shimasaki, H. (1994) Methods Enzymol. 233, 338–346.
[23] Buege, J.A. and Aust, S.D. (1978) Methods Enzymol. 52, 302–310.
[24] Sugioka, K., Nakano, M., Kurashige, S., Akuzawa, Y. and Goto, T. (1986) FEBS Lett. 197, 27–30.
[25] Axllne, S.G. and Reaven, E.P. (1974) J. Cell Biol. 62, 647–659.
[26] Hogg, N., Kalyanaraman, B., Joseph, J., Struck, A. and Parthasarathy, S. (1993) FEBS Lett. 334, 170–174.