Vol. 206, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 5, 1995 Pages 138-145

Induction of Protein Oxidation in Human Low Density Lipoprotein by the Photosensitive Organic Hydroperoxide, N,N'-bis(2-Hydroxyperoxy-2-Methoxyethyl)-1,4,5,8-Naphthalene-Tetra-Carboxylic-Diimide

Seiichi Matsugo[1], Liang-Jun Yan, Derick Han and Lester Packer[2]

Department of Molecular and Cell Biology, University of California at Berkeley, CA 94720-3200

Received November 4, 1994

Abstract: We have developed a new molecular probe, N,N'-bis(2-hydroxyperoxy-2-methoxyethyl)-1,4,5,8-naphthalene-tetra-carboxylic-diimide (NP-III), that specifically generates hydroxyl radical upon irradiation with longer wavelength ultraviolet light (UVA). Hydroxyl radicals are generated only upon irradiation, thus NP-III is a new controllable hydroxyl radical source. Apolipoprotein (apo-B) of human low density lipoprotein (LDL), and bovine serum alubumin (BSA), were irradiated with UVA in the presence of NP-III and their oxidation was evaluated by two independent methods: assay of protein carbonyl groups and gel electrophoresis. NP-III oxidized apo-B and BSA in a time- and concentration-dependent manner. The results demonstrate that NP-III is a contollable, precise, and potentially tagetable source of hydroxyl radicals with which to induce protein oxidation.

Oxidation of LDL has been extensively studied in connection with the biological role of oxidized LDL in aging [1], diabetes [2-4] and other pathological conditions. Oxidation of LDL can be achieved by many active oxygen species including hydroxyl radical [5], superoxide [6-13], singlet oxygen [14-18], and nitric oxide [19-21]. In order to obtain precise and quantitative information concerning LDL oxidation, it is highly desirable to devise a system to induce LDL oxidation with precision. For this purpose, it is necessary to develop a compound that generates a specific active oxygen species quatitatively, under controllable defined conditions. The use of a photochemical reaction is suitable for this purpose. Hence, we have developed a compound that generates hydroxyl radical upon irradiation with longer wavelength ultraviolet (UV) light (> 350 nm); the compound is a hydroperoxide bearing naphthaldiimide chromophore, N,N'-bis(2-hydroxyperoxy-2-methoxyethyl)-1,4,5,8-napthalene-tetra-carboxylic-diimide, (NP-III) [22-25] (Fig. 1). This compound, which has an absorption maximum at 377 nm (ε 27000), generates hydroxyl radical upon irradiation with UVA (320-400 nm); in this sense it can be considered a "photo-Fenton" reagent. It has been shown to cleave DNA at -GG- sites upon irradiation, suggesting the possibility for precise targetting [22, 23]. It is of considerable interest to examine the oxidative damage of LDL and BSA caused by this compound under irradiated conditions.

[1] On leave from Department of Chemical & Biochemical Engineering, Faculty of Engineering, Toyama University, Gofuku 3190, Toyama 930, Japan.

[2] To whom correspondence should be addressed. Fax: 510-642-8313.

0006-291X/95 $5.00

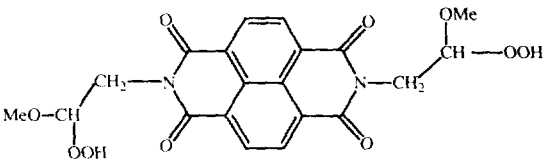


Fig. 1. Structure of NP-III.

Materials and Methods

All reagents used were of reagent grade and used without purification except where indicated. UV spectra were measured using Shimadzu UV 160 U spectrophotometer and irradiation was carried out using an Oriel Corporation 68820 instrument with UVB and UVC cutoff filters. SDS PAGE (Daiichi Multigel, SG611201, 4-20 %) and protein molecular weight markers (SE130024, MW 95000, 68000, 39000, 29000, 20400, 14000) were purchased from Integrated Separation Systems.

Synthesis of NP-III: To a solution of N, N'-(bis-2,2-dimethoxyethyl)-1,4,5,8-naphthalimide (1.11 g, 2.5 mmol) (prepared from the reaction of naphthalene-1,4,5,8-tetracarboxylic anhydride and 2,2-dimethoxyethylamine) in dry methylene chloride (50 ml), was added excess hydrogen peroxide, prepared by extracting 30% aqueous hydrogen peroxide with ether. Triflic acid (0.24 ml, 2.5 mmol) was added to the resulting solution at 0°C by a syringe. This solution was stirred for 1h at 0°C. After the reaction, the reaction mixture was poured into ice water. The methylene chloride layer was separated, and washed with water two times. Evaporation of the methylene chloride layer at 0°C gave yellow solid which was washed with cold ethyl ether to give analytically pure NP-III as a yellow solid.

Preparation of LDL: The density of whole plasma was adjusted to 1.24 gm / ml by adding KBr (0.3816 g / ml plasma). To dissolve KBr, the mixture was stirred gently with a magnetic flea at 4 °C. Eight centrifuge tubes were charged with 27 ml of deaerated (argon or nitrogen) mock buffer (density 1.006, 34.32g NaCl, 0.300g EDTA dissolved in 3l distilled water). Twelve ml of plasma per tube was underlayed using a long luer fitting needle. The tubes were centrifuged in a Vti 50 vertical rotor at 50000 rpm at 10°C for 2 hours. Crude LDL, which appeared as a yellow band in the center of the tube, was removed by aspirating through the side of the tube. From 12 ml of plasma, 3 ml of LDL could be obtained. The crude LDL (25 ml) was cetrifuged at 10°C, 51000 rpm for 24 hours to get pure LDL as a yellow band at the top of the tube. To get rid of the salt effect, EDTA contaminating the LDL sample solution was removed by dialysis against PBS buffer (pH 7.4) for 24 h.

Oxidation of Salicylic Acid by NP-III: In this and in other irradiations NP-III solutions were prepared in acetonitrile to a concentration ten-fold the final sample concentration. Then, to a solution of 250 µl of 2 mM of salicylic acid and 200 µl of 27 mM citrate and 30 mM acetate buffer was added 50ul NP-III to final concentrations of 0,5,10, 25, 50, and 100 µM. Samples were then directly irradiated. A duplicate set of samples covered with foil served as dark controls. Irradiation was provided by an Oriel Corporation 68820 apparatus using UVB and UVC cutoff filters (cutoff wavelength, 320 nm) at a distance of 10 cm. Samples were irradiated for 30 min. After the reaction, the reaction mixture was subjected to HPLC-ECD system to detect the formation of 2,3-dihydroxy-benzoic acid and 2,5-dihydroxy-benzoic acid. The yield for the formation of hydroxylated salicylate were determined by comparing the peak areas to those of standards [26].

BSA Oxidation by NP-III: The concentration of BSA was adjusted to 2 mg/ml in 0.1M sodium phosphate buffer (pH 7.2). NP-III solutions were prepared in acetonitrile to ten-fold their final concentration, as for salicylic acid. Solutions containing 900 µl of BSA and 100 µl of NP-III to final concentrations of NP-III of 0, 1, 5, 10, and 25 µM were directly irradiated as for salicylic acid. A duplicate set of samples covered with foil served as dark controls. The carbonyl content of the BSA was measured as described [27]. Briefly, the reaction mixture was treated with 2,4--dinitrophenylhydrazine at room temperature for 2h. After the reaction the mixed solution was washed with ethyl alcohol : ethyl acetate (1 : 1, v / v) three times. The protein pellet was dissolved in 6 M guanidine-HCl solution (pH 2.3) and the absorbance at 360 nm was measured.

LDL Oxidation by NP-III: LDL oxidation by NP-III at defined concentrations was carried out under almost the same reaction conditions as those for BSA reaction. 900 µl of LDL solution

was added 100 µl of NP-III to final concentrations of NP-III of 1,2, 5, and 10 µM. The protein concentration of LDL was 1 mg/ml, confirmed by the Lowry method [28]. The irradiation was carried in the same manner as with BSA. After the reaction, the sample solution was treated with the method described in the BSA section and the carbonyl content of protein of LDL was determined spectroscopically.

The time-dependent LDL oxidation by 10 and 25 µM NP-III was also studied by removing sample aliquots at various time periods during a 30 minute irradiation.

Gel Electrophoresis of LDL: Gel electrophoresis was carried out using the ISS 250 (Integrated Separation System) power supply and SDS PAGE (4 - 20 % gradient gel). Twenty µl aliquots from the same sample solution of the oxidized LDL as were used for protein carbonyl analysis were treated with an equal volume of commertially available denaturing and reducing reagent (ISS SA 10052). Twenty µl of the sample solutions were loaded on the gel. After fixation by 12 % trichloroacetic acid, the staining of the LDL protein was carried out using the ISS Pro-Blue kit (Integrated Separation System, SE140002).

Results

When NP-III was irradiated with UVA light in the presence of salicylic acid, formation of 2,3- and 2,5-dihydroxy-benzoic acid was observed in a concentration-dependent fashion (Fig. 2). Samples that were not irradiated showed no detectable 2,3- or 2,5-dihydroxy-benzoic acid. Samples with no NP-III that were irradiated for 30 min showed no evidence of hydroxyl radical formation.

When BSA solution was irradiated in the presence of NP-III, protein oxidation was observed. Protein carbonyl concentration was 15 nmol/mg at 1 µM NP-III and increased to 45

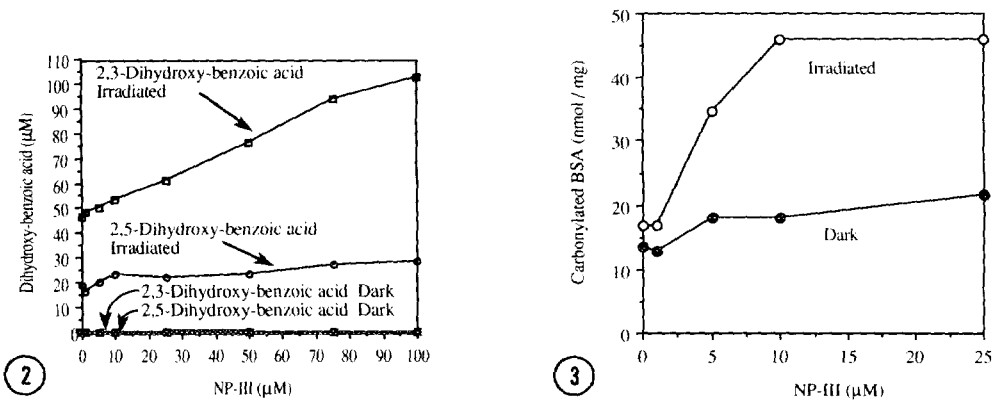
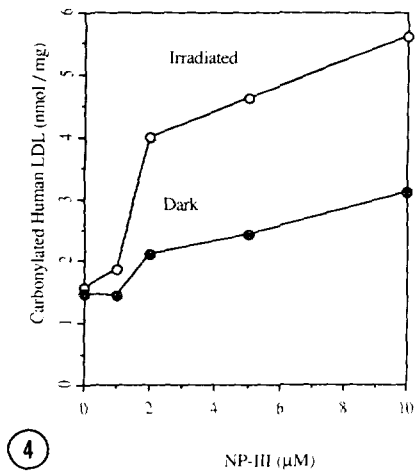


Fig. 2. Oxidation of Salicylic Acid by NP-III. Salicylic acid (2 mM) dissolved in 27 mM citrate and 30 mM acetate buffer was incubated with various concentrations of NP-III. After the irradiation the sample solutions were subjected to HPLC-ECD and the amounts of the hydroxylated salicylic acid were determined by comparing the peak area with those of standards of 2,3- and 2,5-dihydroxy-benzoic acid.

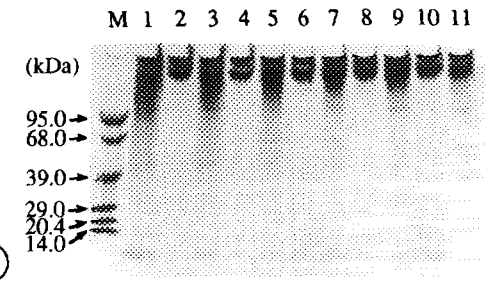
Fig. 3. Oxidation of Bovine Serum Alubumin by NP-III. BSA (2mg / ml) was incubated with various concentrations of NP-III in 0.1 M phosphate buffer solution (pH 7.0). After the irradiation, the sample solution was reacted with 2,4-dinitrophenylhydrazine and the precipitate was washed three times using ethyl alcohol - ethyl acetate (v / v, 1: 1). The protein carbonyl concentration of BSA was determined at 360 nm absorption band in guanidine - HCl solution (pH 2.3).

nmol/mg at 10 μM concentration (Fig. 3). Without photoirradiation the protein carbonyl concentration of BSA remained almost constant, and samples which contained no NP-III and were irradiated for 30 min exhibited almost the same level of protein carbonyls as those held in the dark (about 15 nmol/mg).

LDL oxidation was induced by UVA irradiation in the presenceof NP-III. Upon irradiation for 30 min., the protein carbonyl concentration of LDL increased to 5.6 nmol/mg protein, which is about three times the control value (Fig. 4). The increase in carbonyl concentration was dependent on the concentration of NP-III. Without irradiation, an increase of protein carbonyl concentration was also observed, but the value was much lower than those of photoirradiated at every concentration. Using the same sample solutions as used for protein carbonyl analysis, we performed gel electrophoresis studies. LDL photoreacted with NP-III showed tailing bands, due to the fragmentation of high molecular weight LDL protein, at each concentration examined (Fig.5). The tailing band was not observed in the dark control samples.



④



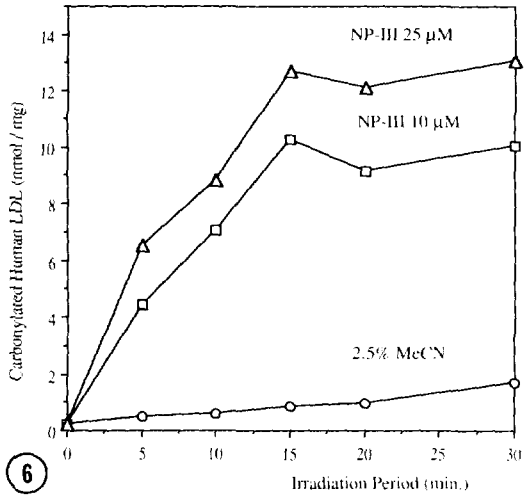
⑤

Fig. 4. Oxidation of LDL by NP-III. Human LDL (1.5 mg protein / ml) was incubated with various concentrations of NP-III in saline solution (pH 7.4). After the photoreaction, the sample solution was reacted with 2,4-dinitrophenylhydrazine and the precipitate produced was washed three times using ethyl alcohol - ethyl acetate (v / v, 1 : 1). The carbonyl concentration of the protein in human LDL was determined by measuring the 360 nm absorption band of the precipitate in guanidine - HCl solution (pH 2.3).

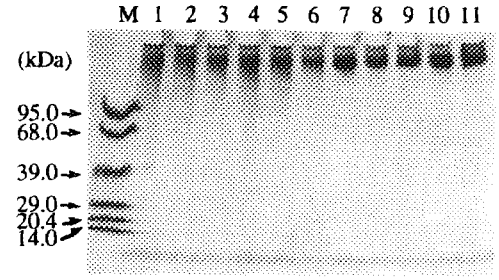
Fig. 5. SDS Gel Electrophoresis of LDL Proteins irradiated with NP-III. The sample solution of 1 part of various concentrations of NP-III, 4 parts of 0.1 M phosphate buffer, and 5 parts of LDL (1.5 mg protein / ml) in saline buffer was irradiated for 30 min. After denaturation of the protein, the reaction mixture was subjected to SDS-PAGE.

M: Protein Molecular Marker; lane 1: 25 μM NP-III with irradiation; lane 2: 25 μM NP-III without irradiation; lane 3: 10 μM NP-III with irradiation; lane 4: 10 μM NP-III without irradiation; lane 5: 5 μM NP-III with irradiation; lane 6: 5 μM NP-III without irradiation; lane 7: 2 μM NP-III with irradiation; lane 8: 2 μM NP-III without irradiation; lane 9: 1 μM NP-III with irradiation; lane 10: 1 μM NP-III without irradiation; lane 11: 2.5 % MeCN with irradiation.

We also examined the oxidative damage of LDL protein by varying the irradiation period of in the presence of a two concentrations of NP-III. Upon irradiation in the presence of NP-III the carbonyl concentrations of protein in LDL increased in a time-dependent fashion up to 15 min of irradiation, in the case of both 10 μM and 25 μM concentration (Fig. 6). The control experiment using 2.5 % of acetonitrile solution at photoirradiated conditions shows that this protein oxidation is induced by NP-III and not by other components of the reaction mixture. The protein carbonyl concentration of LDL reached maximum values of more than 10 nmol / mg, which are several



6



7

Fig. 6. Time course of formation of Protein Carbonyls in Human LDL irradiated in the presence of NP-III. Human LDL (1.5 mg protein / ml) was incubated with 100 μM of NP-III in saline solution (pH 7.4). The photochemical reaction was carried out for the indicated times. After the photoreaction, the sample solution was reacted with 2,4-dinitrophenylhydrazine and the precipitate produced was washed three times using ethyl alcohol - ethyl acetate (v / v, 1 : 1). The carbonyl concentration of the protein in human LDL was determined by measuring the 366 nm absorption band of the preticipate in guanidine - HCl solution (pH 2.3).

Fig. 7. SDS Gel Electrophoresis Studies on the Time-Dependent reaction of hydroxyl radicals produced by NP-III with LDL. The sample solution of the 1 part of 250 μM (lanes 1, 2, 3, 9) or 100 μM (lanes 4, 5, 6, 10) NP-III, 4 parts of 0.1 M phosphate buffer, and 5 parts of LDL (1.5 mg protein / ml) in saline buffer was irradiated for the indicated times, below. After the denaturation of the protein, the reaction mixture was subjected to SDS-PAGE.

M: Protein Molecular Marker; Lane 1: 25 μM NP-III with 30 min. irradiation; Lane 2: 25 μM NP-III with 15 min. irradiation; Lane 3: 25 μM NP-III with 5 min. irradiation; Lane 4: 10 μM NP-III with 30 min. irradiation; Lane 5: 10 μM NP-III with 15 min. irradiation; Lane 6: 10 μM NP-III with 5 min. irradiation; Lane 7: 2.5 % MeCN with 30 min. irradiation; Lane 8: 2.5 % MeCN with 15 min. irradiation; Lane 9: 25 μM NP-III without irradiation; Lane 10: 10 μM NP-III without irradiation; Lane 11: 2.5% MeCN without irradiation.

times higher than that of the control value. The lower initial values and higher final values of protein carbonyls in this sample compared to those for the sample exposed to various concentrations of NP-III may be due to the fact that these were different LDL samples.

To determine whether oxidative damage continued in the dark after illumination ceased, LDL samples, prepared as above with 10 uM NP-III, were illuminated with UVA light for 5 minutes, then part of the sample was covered with aluminum foil while the remainder continued to be illuminated to thrity minutes. The concentration of protein carbonyls in the sample was 5.94 nmol/mg at five minutes, and 7.35 nmol/mg in the sample held an additional 25 min. in the dark compared to 23.5 nmol/mg in the sample which remained illuminated. Hence, oxidative damage to the protein virtually ceased when irradiation ceased.

SDS-PAGE also reflected the varying degrees of oxidation in the time-course samples (Fig. 7). The tailing of high molecular weight protein is obvious in cases of 5, 15, and 30 min. irradiation at 25 μM concentration of NP-III. In the cases of 10 μM concenttration of NP-III, the obvious tailing band was observed in the cases of 15, and 30 min. irradiation. In other cases, no clear tailing bands can be observed at all. This corresponds well with the various protein carbonyl concentrations shown in Fig. 6.

Discussion

NP-III is a new and novel compound that may be useful in study oxidation reactions initiated by hydroxyl radicals. It's main attraction is the precision with which it can be used. It generates almost exclusively hydroxyl radicals and hydroxyl radical generation can be precisely controlled through controlling irradiation with UVA. We have used this compound previously in studies of microsomes [29] and DNA [22-24]. In these studies we extend its use to LDL, a compound whose oxidative modification is thought to be a key step in formation of atherosclerotic plaque [25].

The generation of hydroxyl radical from NP-III was confirmed by detection of the formation of 2,3-dihydroxy-benzoic acid and 2,5-dihydroxy-benzoic acid from the photoreaction of NP-III with salicylic acid by HPLC-ECD method [26]. Based on these results and our previous ESR result [29], the generation of hydroxyl radical from NP-III was confirmed, which prompted us to examine the oxidation of protein and LDL.

Protein oxidation was induced by irradiation of NP-III. The concentration of protein carbonyls (45 nmol/mg) in the BSA sample irradiated in the presence of 10 μM of NP-III compares with that obtained from vanadium-H_2O_2 oxidation [30]. However, protein oxidation induced by hydroxyl radical generated from the vanadium-catalyzed decomposition of hydrogen peroxide required a high concentration of vanadium sulfate (1000 μM) and was not subject to the sort of control that NP-III reaction was; the amount of damage can be limited by limiting the period of irradiation as well as by manipulating the concentration of NP-III.

NP-III also induced oxidation of LDL, as evidenced both by protein carbonyl formation and by SDS-PAGE analysis. In all cases, the degree of tailing on the SDS-PAGE corresponded to protein carbonyl formation, and both degree of protein carbonyl formation and changes on SDS-PAGE could be modulated by either changing the concentration of NP-III (Figs. 4 and 5) or by

changing the time of irradiation (Figs. 6 and 7). In these experiments, protein carbonyl formation virtually stopped when UVA irradiation ceased, compared to samples that remained irradiated. This opens the possibility of manipulating samples between rounds of oxidation, which may be useful, for example, to examine the protecive effects of antioxidants at various stages of protein oxidation. In addition, the structure of NP-III is such that it can potentially form covalent bonds with nucleophilic groups (Fig. 1), hence hydroxyl radical formed on irradiation could be targetted to nearby groups.

In summary, these experiments show that NP-III produces hydroxyl radicals upon irradiation with UVA light, and that it causes oxidative damage, measurable as protein carbonyl formation and changes in electrophoretic mobility, in human LDL. Because its production of hydroxyl radical can be precisely controlled, it is a useful compound for the study of oxidation of LDL and other proteins.

Acknowledgments: This research was supported by the Palm Oil Research Institute of Malaysia (PORIM); Ipsen Institute, Paris, France; and the Council for Tobacco Research Grant # 3346R2.

References

1. Sambrano, G. R., Parthasarathy, S., Steinberg, D. (1994) *Proc. Natl. Acad. Sci. U. S. A.*, **91**, 3265.
2. Jozwiak, Z., Palecz, D., Bartosz, G. (1994) *Biochem. Mol. Biol. Int.*, **32**, 259.
3. Bucala, R., Makita, Z., Koschinsky, T., Cerami, A., Vlassara, H. (1993) *Proc. Natl Acad. Sci. U. S. A.*, **90**, 6434.
4. Taus, M., Ferretti, G., Curatola, G., Dousset, N., Solera, M. L., Valdiguie, P., (1992) *Biochem. Int.*, **28**, 835.
5. Lynch, S. M., Frei, B. (1993) *J. Lipid Res.*, **34**, 1745.
6. Dickson, C., Stern, A. (1990) *Int. J. Biochem.*, **22**, 501.
7. Darley-Usmar, V. M., Hogg, N., O'Leary, V. J., Wilson, M. T., Moncada, S. (1992) *Free Rad. Res. Commun.*, **17**, 9.
8. Thomas, C. E., (1992) *Biochim. Biophys. Acta*, **1128**, 50.
9. Hogg, N., Darley-Usmar, V. M., Wilson, M. T., Moncada, S. (1993) *FEBS Lett.*, **326**, 199.
10. Heinecke, J. W., Kawamura, M., Suzuki, L., Chait, A. (1993) *J. Lipid Res.*, **34**, 2051.
11. Wang, J. M., Chow, S. N., Lin, J. K. (1994) *FEBS Lett.*, **342**, 171.
12. Aviram, M., Rosenblat, M. (1994) *J. Lipid Res.*, **35**, 385.
13. Li, Q., Cathcart, M. K., (1994) *J. Biol. Chem.*, **269**, 17508.
14. Jialal, I., Norkus, E. P., Cristol, L., Grundy, S. M., (1991) *Biochim. Biophys. Acta*, **1086**, 134.
15. Wagner, J. R., Motchnik, P. A., Stocker, R., Sies, H., Ames, B. N. (1993) *J. Biol. Chem.*, **268**, 18502.
16. Ojima, F., Sakamoto, H., Ishiguro, Y., Terao, J. (1993) *Free Rad. Biol. Med.*, **15**, 377.
17. Reaven, P. D., Fergson, E., Navab, M. Powell, F. L. (1994) *Arteriosclerosis and Thrombosis*, **14**, 1162.
18. Jessup, W., Mohr, D., Gieseg, S. P., Dean, R. T., Stocker, R. (1992) *Biochim. Biophys. Acta*, **1180**, 73.
19. Graham, A., Hogg, N., Kalyanaraman, B., O'Leary, V., Darley-Usmar, V., Moncada, (1993) *FEBS Lett.*, **330**, 181.
20. Hogg, N., Kalyanaraman, B., Joseph, J., Struck, A., Parthasarathy, S. (1993) *FEBS Lett.*, **334**, 170.
21. Halliwell, B. (1993) *Haemostasis*, **23**, 118.
22. Matsugo, S., Saito, I. (1990) *Nuc. Acid Res. Symp.*, **22**, 57.
23. Matsugo, S., Yamamoto, Y., Kawanishi, S., Sugiyama, H., Matsuura, T., Saito, I. (1991) *Angew. Chem. Int. Ed. Engl.*, **30**, 1351.

24. Matsugo, S., Kumaki, S., Mori, T., Saito, I. (1993) *Chem. Lett.*, 378.
25. Matsugo, S., Kodaira, K.-I., Saito, I. (1993) *Bioorg. Med. Chem. Lett.*, **3**, 1571.
26. Feix, J. B., Kalyanaraman (1991) *Arch. Biochem. Biophys.*, **15**, 43.
27. Reznick, A. Z., Packer, L. (1994) *Method Enzym.*, **233**, 357.
28. Lowry, O. H., Rosenbrough, H. J., Farr, A. L., Randall, R. J. (1951) *J. Biol. Chem.*, **193**, 265.
29. Konishi, T., Kagan, V. E., Matsugo, S., Packer, L. (1991) *Biochem. Biophys. Res. Commun.*, **175**, 129.
30. Keller, R. J., Halmes, N. C., Hinson, J. A., Pumford N. R.(1993) *Chem. Res. Toxicol.*, **6**, 430.