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Review

Protein oxidation by the cytochrome P450 mixed-function oxidation system

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Abstract

This mini-review summarizes results of studies on the oxidation of proteins and low-density lipoprotein (LDL) by various mixed-function oxidation (MFO) systems. Oxidation of LDL by the $O_2/FeCl_3/H_2O_2$/ascorbate MFO system is dependent on all four components and is much greater when reactions are carried out in the presence of a physiological bicarbonate/CO_2 buffer system as compared to phosphate buffer. However, $FeCl_3$ in this system could be replaced by hemin or the heme-containing protein, hemoglobin, or cytochrome *c*. Oxidation of LDL by the O_2/cytochrome P450 cytochrome *c* reductase/NADPH/$FeCl_3$ MFO system is only slightly higher (25%) in the bicarbonate/CO_2 buffer as compared to phosphate buffer, but is dependent on all components except $FeCl_3$. Omission of $FeCl_3$ led to a 60% loss of activity. These results suggest that peroxymonobicarbonate and/or free radical derivatives of bicarbonate ion and/or CO_2 might contribute to LDL oxidation by these MFO systems.
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As an outgrowth of studies to elucidate the mechanisms involved in the regulation of protein turnover, it was discovered that oxidation of proteins by mixed-function oxidation (MFO) systems targets the proteins for proteolytic degradation [1–13]. Although most amino acid residues of proteins are susceptible to oxidation by MFO systems [14–16], the conversion of some amino acid residues (especially lysine, arginine, and proline residues) to carbonyl derivatives represents a major mechanism of protein oxidation [17,18]. Consequently, the carbonyl content of proteins is the most commonly used measure of MFO oxidation, and a number of highly sensitive methods have been developed for the assay of protein carbonyl derivatives (see [19] for review).

Because many of the enzymes that are particularly sensitive to oxidation were among those that had been shown to accumulate as inactive or less active forms during aging, it was proposed that the age-related accumulation of these enzymes might reflect an age-related increase in the level of reactive oxygen species (ROS) and/or an age-related loss in the enzymes that catalyze the proteolytic degradation of oxidized enzymes [20,21]. This concept is supported by results of studies showing that the level of oxidized proteins (protein carbonyls) increases with animal age [22,23] and that there is an age-related loss in the ability to degrade oxidized proteins [24–26]. The age-related increase in protein oxidation could be due to any one of several different age-related changes as follows: (a) a decrease in proteolytic activities that degrade oxidized proteins, (b) an increase in the rate of ROS generation associated with alterations in mitochondrial electron transport activity and/or a number of environmental factors, and (c) a decrease in one or more of many different antioxidant activities [19,27]. Significantly, an increase in the level of oxidative protein damage is also associated with a number of diseases [27–30].

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Table 1
Homogeneous preparations of phenobarbital-inducible cytochrome P450 2B4 (previously called P450 LM2) from rabbit liver microsomes and NADPH-cytochrome P450 reductase were gifts from M.J. Coon, and purified preparations of *P. putida* cytochrome P450 (P450cam), redoxin reductase, and redoxin were gifts from I.C. Gunsalus

Composition of various mixed-function oxidation systems
(1) H_2O_2/ Fe(III)/ascorbate
(2) O_2/cytochrome-P450 (2B4), from rabbit liver microsomes/cytochrome P450 reductase/NADPH/Fe(III)/dilauroylphosphatidylcholine
(3) O_2/*Pseudomonas putida* cytochrome-P450/redoxin reductase, redoxin/NADH/Fe(III)
(4) O_2/microbial diaphorase/Fe(III)/NADH
(5) Xanthine oxidase/hypoxanthine/ferredoxin or putidaredoxin/Fe(III)

Studies in this laboratory demonstrated that any one of the five MFO systems shown in Table 1 is able to catalyze the inactivation of a number of enzymes [1–10], and that the inactivation is associated with the generation of carbonyl derivatives and conversion of the enzymes to forms that are highly susceptible to degradation by subtilisin [31], the 20S proteasome [8–10], and a protease obtained from *Escherichia coli* [1,2,11]. Significantly, the absence of any one of the components in the various MFO systems led to a substantial decrease in protein modification activity [3–5]. Moreover, all of these MFO systems were inhibited by catalase, by various chelating agents, and by Mn(II) which was shown to inhibit the reduction of Fe(III) to Fe(II) [32,4,5]. Based on these results, it was proposed that the oxidation of proteins involves reduction of Fe(III) to Fe(II) followed by reaction of Fe(II) with H_2O_2 to form the highly reactive hydroxyl radical (reaction 1), which is known to promote oxidation of proteins [14–16].

$$H_2O_2 + Fe(II) \rightarrow {}^{\bullet}OH + OH^- + Fe(III) \quad (1)$$

Because many MFO reactions are not inhibited by $^{\bullet}OH$ radical scavengers, it was proposed that the protein modifications are site-specific processes in which Fe(II) is bound to metal-binding sites on t he protein and then reacts with H_2O_2 to form $^{\bullet}OH$ that reacts preferentially with amino acids at the metal-binding site [33]. Other studies with cytochrome P450 MFO systems shown in Table 1 (systems 2 and 3) confirm that oxidation of various enzymes is stimulated by addition of Fe(III) and that these reactions are inhibited by catalase and therefore probably involve H_2O_2 [1,3–5]. Results of earlier studies [34–37] led to the proposal that the Fe(III) in the heme moiety of Cyt-P450 undergoes one electron reduction to form a Fe(II) derivative that can bind O_2 to form a Cyt-P450-Fe(II)-O_2 complex, which is in equilibrium with the radical derivative Cyt-P450-Fe^{3+}-($O_2^{-\bullet}$). It is believed that these derivatives play an important role in the oxidation of proteins and lipids. However, the mechanism by which H_2O_2 is generated in the P450 systems is not well established. Contrary to our results and results of some other workers [38–40], it was reported that catalase does not inhibit the Cyt-P450-mediated oxidation of microsomal proteins, and that this oxidation is not dependent upon the presence of free Fe(III) nor is it affected by ascorbate [34].

Importance of bicarbonate buffer systems in metal-catalyzed oxidation of proteins and lipids

In earlier studies, we demonstrated that the oxidation of free amino acids to ammonia and α-ketoacids by the H_2O_2/Fe(II) MFO system is absolutely dependent on the presence of sodium bicarbonate and CO_2 [41]. High concentrations of bicarbonate (15–25 mM) and CO_2 (1.3 mM) constitute the major physiological buffer systems in animals; however, because such buffers are difficult to prepare and control, they are rarely used in in vitro studies. Based on this consideration, we compared the effects of sodium bicarbonate/CO_2 buffers and phosphate buffers on the oxidation of low-density lipoprotein (LDL) by the H_2O_2/Fe(II)/ascorbate MFO system [42]. This MFO system has been widely used in studies on LDL oxidation, but only in the presence of non-physiological buffers [43,44]. Results of our studies showed that the time- and MFO-dependent oxidation of LDL, as measured by malondialdehyde (MDA) and protein carbonyl formation, was very much greater when reactions were carried out in bicarbonate buffers (Fig. 1). Moreover, when Fe(II) in this MFO system was replaced by hemin or the heme-containing proteins (hemoglobin or cytochrome *c*), oxidation by H_2O_2 was

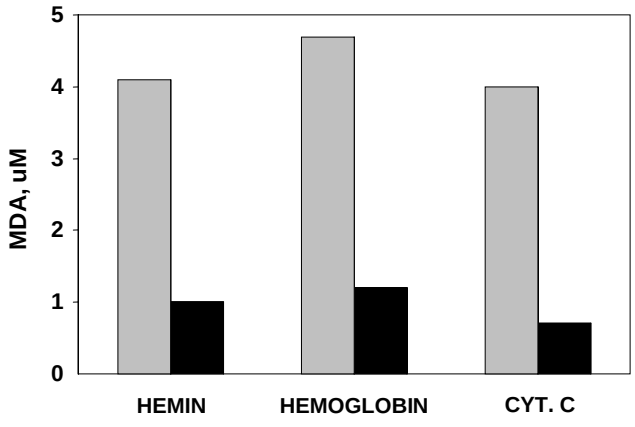

Fig. 1. Comparison of bicarbonate and phosphate buffers on MFO oxidation of low-density lipoprotein. LDL (200 μg protein) was oxidized by MFO systems composed of 100 μM $FeCl_3$, 200 μM H_2O_2, and 50 μM ascorbate (AS), in air (O_2), as indicated. After incubation for 8 and 16 h, at 37 °C, samples were assayed for MDA and protein carbonyl content, as previously described [42]. This is a replot of some data in Fig. 1 of [42].

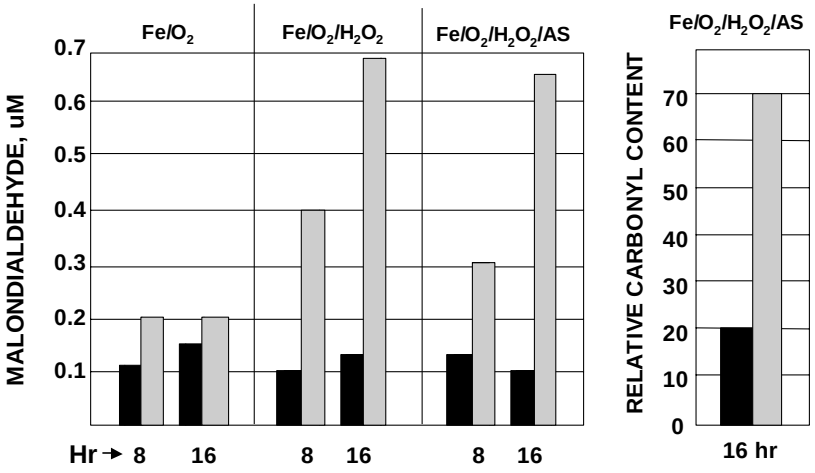

Fig. 2. Comparison of bicarbonate and phosphate buffers on the oxidation of LDL by hydrogen peroxide and heme-containing substances. Mixtures containing 200 μg LDL protein, 200 μM H_2O_2, 50 μM ascorbate, and either 2 μM hemin, 1 μM hemoglobin, or 10 μM cytochrome *c*, as indicated, were incubated at pH 7.6, in phosphate buffer (black bands) or bicarbonate buffer (gray bands). After 8 h incubation at 37 °C, the concentration of MDL was measured as previously described [42]. This is a replot of some data in Fig. 7 of [42].

4- to 5-fold greater in bicarbonate buffer than in phosphate buffer (Fig. 2). Interestingly, the oxidation of LDL by the Fe(II)/H_2O_2/ascorbate system was 4-fold greater in the presence of 400 μM ADP [42] and 7-fold greater by 400 μM ATP (unpublished results) when the reactions were carried out in bicarbonate buffer; whereas ADP and ATP had no effect on reactions in phosphate buffer.

Cyt-P450-mediated oxidation of LDL in bicarbonate/CO_2 and phosphate buffers

Based on the results described above, we compared the effects of phosphate and bicarbonate/CO_2 buffers on the oxidation of LDL by the P-450 LM2 MFO system (system 2, Table 1). Results of preliminary experiments summarized in Fig. 3 show that formation of protein carbonyl derivatives is significantly greater when reactions are carried out in the bicarbonate/CO_2 buffer compared to phosphate buffer. Consistent with results of previous studies on the oxidation of various proteins (enzymes) by the P-450 LM2 system [3–5], the oxidation of LDL to carbonyl derivatives is dependent on the presence of cytochrome-*c*-reductase and NADPH. However, the conversion of LDL to carbonyl derivatives was less dependent on the addition of Fe(III). Carbonyl formation in the absence of added Fe(III) was about 40% of values observed in the presence of added Fe(III) in both buffer systems (Fig. 3). Moreover, in contrast to the oxidation of microsomal proteins [34], this iron-independent generation of carbonyl derivatives was only slightly inhibited by ascorbate (data not shown).

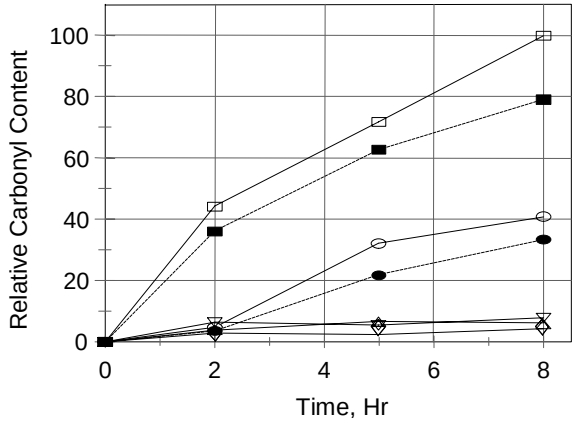

Fig. 3. Effects of bicarbonate and phosphate buffers on the oxidation of LDL by the Cyt-P450 MFO system. The complete system (1 ml) contained 200 μg LDL, 1 μM Cyt P450 reductase, 0.5 μM Cyt P450, 1 mM NADPH, 50 μM $FeCl_3$, and either 14.7 mM bicarbonate/CO_2 buffer (□) or phosphate buffer (■) at pH 7.4. The symbols (○) and (●) refer to reactions in the absence of $FeCl_3$ in bicarbonate and phosphate buffer, respectively. Other symbols refer to reactions in bicarbonate buffer, in the absence of both $FeCl_3$ and either P450 (△), P450 reductase (▽), or NADPH (◇). After incubation in air, 37 °C, for the times indicated, the relative protein carbonyl content was measured as previously described [42].

Discussion

Oxidation of LDL by the H_2O_2/Fe(II)/ascorbate and the Cyt-P450/cytochrome-*c*-reductase/NADPH/Fe(III)/O_2 MFO systems is significantly greater when the reactions are carried out in bicarbonate buffer than in phosphate buffer. These results and earlier studies showing that oxidation of free amino acids by the Fe(II)/H_2O_2 system is almost completely dependent on the presence of bicarbonate/CO_2 [41] suggest the possibility that peroxymonocarbonate [45,46] and/or free radical derivatives of bicarbonate and CO_2 [46–49] may be involved. In contrast to earlier studies showing that oxidative inactivation of several enzymes by the Cyt-P450 MFO system is strongly dependent on the addition of Fe(II) [3–5], results summarized here show that oxidation of LDL by the Cyt-P450 MFO system in the absence of added iron is about 40% of the value obtained in the presence of iron. In view of the fact that heme-containing proteins are able to replace

free iron in the oxidation of LDL by the ascorbate MFO system (Fig. 2), the ability of the P450 MFO system to catalyze oxidation of LDL in the absence of added Fe(III) may reflect ability of the Cyt-P450 heme component to mediate LDL oxidation, as was previously suggested [34].

The mechanisms involved in the ADP and ATP stimulation of oxidation reactions by various MFO systems are ill defined. The observation that ADP stimulates the oxidation of LDL by the ascorbate/Fe(II)/H_2O_2 MFO system in bicarbonate buffer but not in phosphate buffer [42] is at odds with results showing that ADP-Fe(III) stimulates the oxidation of microsomal proteins by the NADPH/Cyt-P450/reductase MFO system when reactions are carried out in phosphate [34] or Tris [35] buffers. It is well established that the Cyt-P450 MFO system is able to reduce ADP-Fe(III) to ADP-Fe(II), but the effect of bicarbonate/CO_2 buffer on this reduction remains to be explored.

References

[1] C.N. Oliver, R.L. Levine, E.R. Stadtman, Regulation of glutamine synthetase degradation, in: H. Holzer (Ed.), Metabolic Interconversion of Enzymes, Springer-Verlag, Berlin, 1981, pp. 259–268.

[2] R.L. Levine, C.N. Oliver, R.M. Fulks, E.R. Stadtman, Turnover of bacterial glutamine synthetase: oxidative inactivation precedes proteolysis, Proc. Natl. Acad. Sci. USA 78 (1981) 2120–2124.

[3] C. Oliver, L. Fucci, R. Levine, M. Wittenberger, E.R. Stadtman, Inactivation of key enzymes by P450-linked mixed function oxidation systems, in: E. Heitanen, M. Laitinen, O. Hannienen (Eds.), Cytochrome P-450 Biochemistry Biophysics and Environmental Implications, Elsevier Biomedical Press, BV, 1982, pp. 531–538.

[4] C.N. Oliver, R.L. Levine, E.R. Stadtman, Regulation of glutamine synthetase degradation, in: L.N. Ornston, S.G. Sligar (Eds.), Experiences in Biochemical Perception, Academic Press, New York, 1982, pp. 233–249.

[5] L. Fucci, C.N. Oliver, M.J. Coon, E.R. Stadtman, Inactivation of key metabolic enzymes by mixed-function oxidation systems, Proc. Natl. Acad. Sci. USA 80 (1983) 1521–1525.

[6] A.J. Rivett, Preferential degradation of the oxidatively modified form of glutamine synthetase by intracellular mammalian proteases, J. Biol. Chem. 260 (1985) 300–305.

[7] E.R. Stadtman, M. Wittenberger, Inactivation of *Escherichia coli* glutamine synthetase by xanthine oxidase, nicotinate hydroxylase, horseradish peroxidase or glucose oxidase: effects of ferredoxin, putidaredoxin, and menadione, Arch. Biochem. Biophys. 239 (1985) 329–379.

[8] A.J. Rivett, J.E. Roseman, C.N. Oliver, R.L. Levine, E.R. Stadtman, Covalent modification of proteins by mixed-function oxidation: recognition by intracellular proteases, in: E.A Khairallah, J.S. Bond, I.W.C. Bird (Eds.), Intracellular Protein Catabolism, Alan R. Liss, New York, 1985, pp. 317–328.

[9] A.J. Rivett, The effect of mixed-function oxidation of enzymes on their susceptibility to degradation by a nonlysomal cysteine proteinase, Arch. Biochem. Biophys. 243 (1985) 624–632.

[10] A.J. Rivett, Regulation of intracellular protein turnover: covalent modification as a mechanism of marking proteins for degradation, Curr. Top. Cell. Reg. 28 (1986) 291–337.

[11] J.E. Roseman, R.L. Levine, Purification of a protease from *Escherichia coli* with specificity for oxidized glutamine synthetase, J. Biol. Chem. 262 (1987) 2101–2110.

[12] R.A. Dunlop, K.J. Rogers, R.T. Dean, Recent developments in the intracellular degradation of oxidized proteins, Free Rad. Biol. Med. 33 (2002) 894–906.

[13] R.T. Dean, S. Fu, R. Stocker, M.J. Davies, Biochemistry and pathology of radical-mediated protein oxidation, Biochem. J. 324 (1997) 1–18.

[14] W.M. Garrison, M.E. Jayko, W. Bennett, Radiation-induced oxidation of protein in aqueous solution, Radiat. Res. 16 (1962) 483–502.

[15] W.M. Garrison, Reaction mechanisms in the radiolysis of peptides, polypeptides, and proteins, Chem. Rev. 87 (1987) 381–398.

[16] A.J. Swallow, Effect of ionizing radiation on proteins. RCO groups peptide bond cleavage, inactivation, –SH oxidation, in: A.J. Swallow (Ed.), Radiation Chemistry of Organic Compounds, Pergamon Press, New York, 1960, pp. 211–224.

[17] A. Amici, R.L. Levine, L. Tsai, E.R. Stadtman, Conversion of amino acid residues in proteins and amino acid homopolymers to carbonyl derivatives by metal-catalyzed reactions, J. Biol. Chem. 264 (1989) 3341–3346.

[18] J.R. Requena, C.C. Chao, R.L. Levine, E.R. Stadtman, Glutamic and aminoadipic semialdehydes are the main carbonyl products of metal-catalyzed oxidation of proteins, Proc. Natl. Acad. Sci. USA 98 (2001) 69–74.

[19] E.R. Stadtman, Role of oxidant species in aging, Curr. Med. Chem. 11 (2004) 1105–1112.

[20] C.N. Oliver, R. Fulks, R.L. Levine, L. Fucci, A.J. Rivett, A.J. Roseman, E.R. Stadtman, Oxidative inactivation of key metabolic enzymes during aging, in: A.K. Roy, B. Chatterjee (Eds.), Molecular Basis of Aging, Academic Press, New York, 1984, pp. 235–260.

[21] C.N. Oliver, B. Ahn, M.E. Wittenberger, R.L. Levine, E.R. Stadtman, Age-related alterations of enzymes may involve mixed-function oxidation reactions, in: R.E. Adelman, E.E. Dekker (Eds.), Modification of Proteins During Aging, Alan R. Liss, New York, 1985, pp. 39–52.

[22] C.N. Oliver, B.-W. Ahn, E.J Moerman, S. Goldstein, E.R. Stadtman, Age-related changes in oxidized proteins, J. Biol. Chem. 262 (1987) 5488–5491.

[23] C.N. Oliver, R.L. Levine, E.R. Stadtman, A role of mixed-function oxidation reactions in the accumulation of altered enzyme forms during aging, Geriatr. Biosci. 35 (1987) 947–956.

[24] P.E. Starke-Reed, C.N. Oliver, Protein oxidation and proteolysis during aging and oxidative stress, Arch. Biochem. Biophys. 275 (1989) 559–567.

[25] J.M. Carney, P.E. Starke-Reed, C.N. Oliver, R.W. Landum, M.S. Cheng, J.F. Wu, R.A. Floyd, Reversal of age-related increase in brain protein oxidation, decrease in enzyme activity and loss in temporal and spatial memory by chronic administration of the spin-trapping compound *N-tert*-butyl-α-phenylnitrone, Proc. Natl. Acad. Sci. USA 88 (1991) 3633–3636.

[26] P.A Szweda, B. Friguet, L.I. Szweda, Proteolysis, free radicals, and aging, Free Rad. Biol. Med. 33 (2002) 29–36.

[27] B.S. Berlett, E.R. Stadtman, Protein oxidation in aging, disease, and oxidative stress, J. Biol. Chem. 272 (1997) 20313–20316.

[28] E.R. Stadtman, B.S. Berlett, Reactive oxygen-mediated protein oxidation in aging and disease, Chem. Res. Toxicol. 10 (1997) 485–494.

[29] D.A. Butterfield, E.R. Stadtman, Protein oxidation processes in aging brain, in: P.S. Timiras, E.E. Bittar (Eds.), Advances in Cell Aging and Gerontology, JAI Press, 1997, pp. 161–191.

[30] E.R. Stadtman, R.L. Levine, Protein oxidation, Ann. N. Y. Acad. Sci. 899 (2000) 191–208.

[31] J.M. Farber, R.L. Levine, Oxidative modification of glutamine synthetase of *E. coli* enhances its susceptibility to proteolysis, Fed. Proc. 41 (1982) 865 (abstract).

[32] K. Nakamura, C. Oliver, E.R. Stadtman, Inactivation of glutamine synthetase by a purified rabbit liver microsomal cytochrome P-450 system, Arch. Biochem. Biophys. 240 (1989) 319–329.

[33] E.R. Stadtman, Role of oxidized amino acids in protein breakdown and stability, Methods Enzymol. 258 (1995) 379–393.

[34] C.K. Mukhopadhyay, I.B. Chatterjee, NADPH-initiated cytochrome P450-mediated free metal ion-independent oxidative damage of microsomal proteins, J. Biol. Chem. 269 (1994) 13390–13397.
[35] A. Sevanian, K. Nordenbrand, E. Kim, L. Ernster, P. Hochstein, Microsomal lipid peroxidation: the role of NADPH-cytochrome P450 reductase and cytochrome P450, Free Rad. Biol. Med. 8 (1990) 145–152.
[36] T.D. Porter, M.J. Coon, Cytochrome P-450: multiplicity of isoforms, substrates, and catalytic and regulatory mechanisms, J. Biol. Chem. 266 (1991) 13469–13472.
[37] B. Halliwell, J.M.C. Gutteridge, Cytochrome P450, in: B. Halliwell, J.M.C. Gutteridge (Eds.), Free Radicals in Biology and Medicine, Oxford University Press, 1999, pp. 14–17.
[38] M. Aviram, U.M. Kent, P.F. Hollenberg, Microsomal cytochrome P450 catalyzes the oxidation of low density lipoprotein, Atherosclerosis 143 (1999) 253–260.
[39] A. Sevanian, F. Ursini, Lipid peroxidation in membranes and low-density lipoproteins: similarities and differences, Free Rad. Biol. Med. 29 (2000) 306–311.
[40] N. Lambert, S.J. Chambers, G.W. Plumb, G. Williamson, Human cytochrome P450s are pro-oxidants in iron/ascorbate-initiated microsomal lipid peroxidation, Free Rad. Res. 24 (1996) 177–185.
[41] E.R. Stadtman, B.S. Berlett, Fenton chemistry: amino acid oxidation, J. Biol. Chem. 266 (1991) 17201–17211.
[42] H. Arai, B.S. Berlett, P.B. Chock, E.R. Stadtman, Effect of bicarbonate on iron-mediated oxidation of low-density lipoprotein, Proc. Natl. Acad. Sci. USA 102 (2005) 10472–10477.
[43] J.W. Heinecke, Oxidants and antioxidants in pathogenesis of atherosclerosis: implications for oxidized low density lipoprotein hypothesis, Atherosclerosis 141 (1998) 1–15.
[44] R. Stocker, J.F. Keaney Jr., Role of oxidative modifications in atherosclerosis, Physiol. Rev. 84 (2004) 1381–1478.
[45] D.E. Richardson, C.A. Regino, H. Yao, J.V. Johnson, Methionine oxidation by peroxymonocarbonate, a reactive oxygen species formed from CO_2/bicarbonate and hydrogen peroxide, Free Rad. Biol. Med. 35 (2003) 1538–1550.
[46] M.G. Bonni, S. Miyamoto, P.D. Mascio, O. Augusto, Production of the carbonate radical anion during xanthine oxidase turnover in the presence of bicarbonate, J. Biol. Chem. 279 (2004) 51836–51843.
[47] M.G. Bonini, D.C. Fernandes, O. Augusto, Albumin oxidation to diverse radicals by the peroxidase activity of Cu,Zn-superoxide dismutase in the presence of bicarbonate or nitrite: diffusible radicals produce cysteinyl and solvent-exposed and -unexposed tyrosyl radicals, Biochemistry 43 (2004) 344–351.
[48] S. Goldstein, G. Czapski, J. Lind, G. Merenyi, Carbonate radical ion is the only observable intermediate in the reaction of peroxynitrite with CO_2, Chem. Res. Toxicol. 14 (2001) 1273–1276.
[49] B. Halliwell, J.M.C. Gutteridge, Radical chemistry: thermodynamics and kinetics, in: B. Halliwell, J.M.C. Gutteridge (Eds.), Free Radicals in Biology and Medicine, Oxford University Press, Oxford, 1999, pp. 46–47.