Effect of bicarbonate on iron-mediated oxidation of low-density lipoprotein

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Oxidation of low-density lipoprotein (LDL) may play an important role in atherosclerosis. We studied the effects of bicarbonate/CO2 and phosphate buffer systems on metal ion-catalyzed oxidation of LDL to malondialdehyde (MDA) and to protein carbonyl and MetO derivatives. Our results revealed that LDL oxidation in mixtures containing free iron or heme derivatives was much greater in bicarbonate/CO2 compared with phosphate buffer. However, when copper was substituted for iron in these mixtures, the rate of LDL oxidation in both buffers was similar. Iron-catalyzed oxidation of LDL was highly sensitive to inhibition by phosphate. Presence of 0.3–0.5 mM phosphate, characteristic of human serum, led to 30–40% inhibition of LDL oxidation in bicarbonate/CO2 buffer. Iron-catalyzed oxidation of LDL to MDA in phosphate buffer was inhibited by increasing concentrations of albumin (10–200 μM), whereas MDA formation in bicarbonate/CO2 buffer was stimulated by 10–50 μM albumin but inhibited by higher concentrations. However, albumin stimulated the oxidation of LDL proteins to carbonyl derivatives at all concentrations examined in both buffers. Conversion of LDL to MDA in bicarbonate/CO2 buffer was greatly stimulated by ADP, ATP, and EDTA but only when EDTA was added at a concentration equal to that of iron. At higher than stoichiometric concentrations, EDTA prevented oxidation of LDL. Results of these studies suggest that interactions between bicarbonate and iron or heme derivatives leads to complexes with redox potentials that favor the generation of reactive oxygen species and/or to the generation of highly reactive CO2 anion or bicarbonate radical that facilitates LDL oxidation.

It has been suggested that the oxidation of low-density lipoprotein (LDL) in the vessel wall may be an early event in atherosclerosis (1–5). Some epidemiological and histochemical studies suggest that metal ion-catalyzed reactions might be involved in LDL oxidation, but these data are controversial (1, 5–8).

The intracellular and serum concentrations of bicarbonate anion (HCO3-) in humans are in the range of 14.7–25 mM and are in equilibrium with 5% CO2 (1.3 mM) to yield pH values of 7.2 and 7.4, respectively. As such, they constitute the major physiological buffers. A number of in vitro studies have been carried out to examine the effects of transition metal ions and physiological iron-containing substances (hemoglobin and others) on the oxidation of LDL. However, these studies have been carried out in nonphysiological buffers, such as phosphate. Previous studies (9–12) have shown that oxidation of free amino acids by metal ion-catalyzed oxidation systems is greatly stimulated by bicarbonate/CO2 buffers. In this work, we compared the effects of bicarbonate/CO2 buffers with phosphate buffer systems on the metal ion-catalyzed oxidation of LDL to malondialdehyde (MDA) and protein carbonyl and methionine sulfoxide (MetO) derivatives.

Materials and Methods

Materials. Hemin, human methemoglobin, human recombinant albumin, and bovine heart cytochrome c were obtained from Sigma. The 2,4-dinitrophenylhydrazine was purchased from Mallinkrodt. The 2,2'-azobis(2-amidinopropane) dihydrochloride was supplied from Wako Pure Chemical (Osaka). All other chemicals were of reagent grade. LDL (density 1.019–1.063 g/ml) was separated from fresh human plasma, obtained from a healthy volunteer with informed consent after fasting for 8 h, by the ultracentrifugation method (13).

Metal Ion-Catalyzed Oxidation of LDL and Analysis of Lipid Peroxidation. Bicarbonate/CO2 buffer containing 23.3 mM bicarbonate and 100 mM NaCl (pH 7.6) was prepared by treating the buffer with chelating resin (Chelex 100, Sigma) and equilibrated by bubbling with 5% CO2/95% air at 37°C just before use. For the metal ion-catalyzed experiments, LDL (200 μg of protein) was dispersed in 1 ml of bicarbonate/CO2 buffer and preincubated in 5% CO2/95% air at 37°C for 30 min. The LDL oxidation was initiated by adding either FeCl2 (100 μM), hemin (2 μM), hemoglobin (1 μM), cytochrome c (10 μM), or CuCl2 (1 μM) in the presence or absence of ascorbate (50 μM), H2O2 (200 μM), ADP (100 or 400 μM), EDTA (100 or 400 μM), deferoxamine (100 or 400 μM), or albumin (10–200 μM). The comparative experiments were carried out in phosphate buffer (23.3 mM, pH 7.6) containing 100 mM NaCl and equilibrated with 100% air at 37°C. At the indicated times, the pH of the reaction mixture was checked, and the concentration of MDA generated by the decomposition of lipid hydroperoxides in the reaction mixture was determined by HPLC monitoring changes in fluorescent intensity at 553 nm with excitation wavelength set at 515 nm (14).

Protein Carbonyl Analysis. Oxidized LDL was derivatized with 2,4-dinitrophenylhydrazine and subjected to SDS/PAGE in a 4–15% gradient acrylamide slab gel. The 2,4-dinitrophenylhydrazine-derivatized proteins were detected by immunoblotting with rabbit anti-dinitrophenyl antisera (DakoCytomation, Glostrup, Denmark) as described in ref. 15. Immunoreactive species were visualized by using the Odyssey infrared imaging system (Li-Cor, Lincoln, NE) with IRDye 800-conjugated donkey anti-rabbit IgG (Rockland, Gilbertsville, PA) as a secondary Ab.

Determination of MetO. Oxidized LDL was treated with CNBr, which cleaves peptide bonds on the carboxyl side of Met to yield homoserine (16). When Met is oxidized to MetO, CNBr fails to cleave such bonds (17). Samples with and without CNBr treatment were hydrolyzed by hydrochloric acid, and the resulting amino acid composition was analyzed by using HPLC techniques (18). The amount of MetO was calculated from the difference in peak areas obtained by integrating the corresponding peaks.

Peroxy Radical-Mediated Oxidation of LDL. LDL (100 μg of protein) in 23.3 mM bicarbonate/CO2 or phosphate buffer (pH 7.6) described above was oxidized by 500 μM 2,2'-azobis(2-amidinopropane) dihydrochloride, a hydrophilic peroxy radical generator, at 37°C in the presence of 100 μM diethylenetri-
ate substantial increases in the rate of MDA formation in bicarbonate after oxidation of LDL by H2O2 in the presence or absence of iron (data not shown).
(100–200 μM), albumin inhibited significantly the MDA formation in bicarbonate/CO₂ buffer. At all concentrations examined (10–200 μM), albumin inhibited the formation of MDA in phosphate buffer in a dose-dependent manner (light gray bars).

It is noteworthy that addition of 200 μM albumin had no effect on the pH of either buffer. To examine the possibility that inhibition of MDA formation by high concentrations of albumin is due to preferential reaction of the reactive oxygen species with albumin protein, we examined the effects of albumin concentration on the generation of protein carbonyl derivatives by the Fe(II)/ascorbate/H₂O₂ system (Fig. 5E) in mixtures containing albumin alone (Right) and in mixtures containing both LDL and albumin (Center). It is evident that the oxidation of albumin in both mixtures increases with increasing albumin concentration in both bicarbonate/CO₂ (lanes 2, 4, 6, and 8) and phosphate buffers (lanes 1, 3, 5, and 7). It is also evident from a comparison of results shown in Fig. 5E Right and Center that LDL stimulates the oxidation of albumin as indicated by the enhanced amount of protein carbonyl derivatives of protein fragments derived from albumin (Mₙ < 200 kDa), whereas albumin had little effect on the oxidation of LDL proteins (proteins of >200 kDa).

**Effect of Iron Chelators on LDL Oxidation.** Oxidation of LDL to MDA by the Fe(II)/ascorbate/H₂O₂ system is stimulated severalfold by the presence of ADP when added at a concentration equivalent to the concentration of FeCl₂ (100 μM) and even much more so by the addition of 400 μM ADP when reactions are carried out in bicarbonate/CO₂ buffer (pH 7.6) but not in phosphate buffer (Fig. 6A). In contrast, addition of 100 μM of EDTA to the oxidation system leads to a great enhancement of MDA formation in both bicarbonate/CO₂ and phosphate buffers, but at 400 μM there is little or no effect of EDTA on MDA generation in either buffer (Fig. 6B). Moreover, when reactions were carried out in the absence of ascorbate, addition of 400 μM...
ADP had no effect in either buffer system, and stimulation of MDA formation by 100 μM EDTA was considerably lower in both buffers (data not shown). Results of other studies showed that, when added at either 100 or 400 μM concentrations, deferoxamine inhibited the oxidation of LDL to MDA (data not shown). Addition of EDTA at a concentration equal to the iron concentration led to a great increase in protein carbonyl content of LDL in both buffer systems (Fig. 6C, lanes 5 and 6). However addition of ADP at four times the iron concentration led to much greater stimulation of protein oxidation in bicarbonate buffer (lane 4) than in phosphate buffer (lane 2), compared with the controls (lanes 1 and 2). We also tested the effect of ATP (400 μM) on the oxidation of LDL by Fe(II)/ascorbate/H2O2 system. ATP strongly stimulated MDA formation and the MDA generated was more than twice to that of observed with ADP after a 4-h incubation (data not shown).

**Oxidation of LDL by Hemin and Heme-Containing Proteins.** Oxidation of LDL to MDA and protein carbonyl derivatives occurs also when FeCl2 in the ascorbate/H2O2 mixture is replaced by hemin, hemoglobin, or cytochrome c. Oxidation in the presence of any one of these heme compounds is greater in bicarbonate/CO2 buffer than in phosphate buffer (Fig. 7). In the absence of ascorbate LDL oxidations by hemin or hemoglobin were slightly decreased, but oxidation by cytochrome c in bicarbonate/CO2 buffer was about the same as that observed in phosphate buffer (data not shown).

**Oxidation of LDL by Peroxyl Radicals.** Oxidation of LDL by peroxyl radicals generated during incubation with 2,2'-azobis(2-aminopropane) dihydrochloride in aqueous solution leads to formation of cholesteryl ester hydroperoxide in both bicarbonate/CO2 and phosphate buffers (Fig. 8).

**Oxidation of LDL by Copper.** In contrast to results obtained in the oxidation of LDL by various iron-dependent systems, the formation of MDA by copper(II) was the same in both bicarbonate/CO2 and phosphate buffers. The rate of Cu(II)-dependent oxidation was greatly increased by the presence of only H2O2 (Fig. 9B). However, when Cu(II) (Fig. 9A) or a mixture of Cu(II), ascorbate, and H2O2 (Fig. 9C) was used to oxidize LDL, an ~4-h lag phase was observed before the large increase in MDA formation.

**Discussion**

Bicarbonate and/or CO2 has been shown to be implicated in several physiological processes as follows: (i) free radical generation from hydrogen peroxide catalyzed by copper/zinc superoxide dismutase (20–23); (ii) peroxynitrite-dependent nitration of protein Tyr residues (24); (iii) the ability of

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**Fig. 6.** Effect of iron chelators and buffer composition on iron-mediated oxidation of LDL. MDA formation in LDL oxidized by Fe(II)/ascorbate/H2O2 system for 16 h at pH 7.6, 37°C, in the presence of either ADP (A) or EDTA (B). The data in A and B were obtained in the presence of 400 μM ADP or EDTA in bicarbonate/CO2 buffer (a) or phosphate buffer (c) or in the presence of 100 μM ADP or EDTA in bicarbonate/CO2 buffer (b) or phosphate buffer (c). (C) Protein carbonyl formation in LDL oxidized by Fe(II)/ascorbate/H2O2 system for 16 h at pH 7.6 was analyzed by Western blot as described in Fig. 1. Protein (170 ng) was used for all lanes in Left and 57 ng of protein was used for all lanes in Right. The oxidation was carried out in the presence or absence of 400 μM ADP or 100 μM EDTA as indicated in either phosphate buffer (lanes 1, 3, 5, and 7) or bicarbonate/CO2 buffer (lanes 2, 4, 6, 8).

**Fig. 7.** Oxidation of LDL by hemin and heme iron proteins in bicarbonate and phosphate buffers. MDA was determined after oxidation of LDL (200 μg protein) in mixtures containing 50 μM ascorbate, 200 μM H2O2, and either 2 μM hemin (A), 1.0 μM hemoglobin (B), or 10 μM cytochrome c (C) in phosphate buffer (a), or bicarbonate/CO2 buffer (b), at pH 7.6. (D–F) After oxidation, the protein carbonyl content of these mixtures was determined by Western blot, as described in Fig. 1, after oxidation mediated by hemin (D), hemoglobin (E), and cytochrome c (F). Lanes 1 and 2 show results obtained in phosphate and bicarbonate/CO2 buffers, respectively.

**Fig. 8.** Peroxyl radical-mediated oxidation of LDL in bicarbonate and phosphate buffers. LDL was oxidized by 500 μM 2,2'-azobis(2-aminopropane) dihydrochloride in phosphate buffer (c) or bicarbonate/CO2 buffer (a), with pH 7.6 at 37°C, in the presence of 100 μM diethylenetriaminepentaacetic acid. Cholesteryl ester hydroperoxide was determined by HPLC with UV (235 nm) detection.
Metal ions are believed to contribute to oxidative stress. Bicarbonate stimulates Trp oxidation and formation of 3-nitrotyrosine on the nitration and oxidation of LDL by peroxynitrite, whereas Ferroni et al. (26) reported that bicarbonate inhibits the oxidation of Lys and Trp residues in LDL by peroxynitrite, whereas Ferroni et al. (27) reported that bicarbonate stimulates Trp oxidation and formation of 3-nitrotyrosine, but inhibits lipid oxidation by peroxynitrite. Although metal ions are believed to contribute to oxidative stress in vivo (28), little or no information is available on a role of metal ions in LDL oxidation in bicarbonate/CO₂ buffers. Therefore, we examined the effects of Fe(II), Cu(II), and some heme-iron derivatives is much greater when reactions are carried out in bicarbonate/CO₂, while the iron-mediated generation of MDA, but at higher concentrations (up to twice the level of iron) albumin inhibited MDA formation (Fig. 5A–D). However, albumin exhibits no effect on iron-dependent oxidation to carbonyl derivatives in both bicarbonate/CO₂ and phosphate buffers (Fig. 5E). To the contrary, as shown in Fig. 5, LDL enhances the formation of albumin carbonyl derivatives. This result could reflect secondary reactions of albumin with LDL-derived lipid peroxides or MDA (35). The observed increase in carbonyls in reactions containing albumin in the absence of LDL suggests that inhibition of MDA formation by albumin in phosphate buffer and by high concentrations of albumin in bicarbonate/CO₂ buffer reflects preferential oxidation of albumin by reactive oxygen species generated by the iron-mediated oxidation system.

Stress-induced hemolysis of circulating erythrocytes leads to the release of hemoglobin (36). Both hemoglobin and its iron-containing degradation product, hemin, have been shown to facilitate LDL oxidation in the presence of H₂O₂ in vitro (37–41). Upon incubation with high concentrations of H₂O₂ in vitro, hemoglobin is degraded to form heme and free iron (28). In view of these findings, we carried out studies to compare the abilities of heme, hemoglobin, and cytochrome c to oxidize LDL in bicarbonate/CO₂ and phosphate buffers. We found that all three heme compounds catalyze the conversion of LDL to MDA and to protein carbonyl derivatives and that these oxidations were much greater in bicarbonate/CO₂ buffer than in phosphate buffer (Fig. 7). It remains to be determined whether the observed effects are related to the release of free iron or heme compounds.

The demonstration that there is almost no difference between bicarbonate/CO₂ and phosphate buffers on the oxidation of LDL by peroxyl radicals (Fig. 8) discounts the possibility that the stimulation of LDL oxidation by iron is due to differences in rates of oxidation by alkyl peroxide, but does not preclude the possibility that bicarbonate stimulates the ability of Fe(II) to generate formation of alkyl peroxides during LDL oxidation.

Finally, it is evident that the oxidation of LDL by Cu(II) is not significantly affected by the buffer composition but is dependent on H₂O₂ and is retarded by ascorbate (Fig. 9).
Results of these studies suggest that interactions between bicanonate and iron or heme derivatives lead to complexes that have redox potentials that favor the generation of reactive oxygen species and/or to the generation of highly reactive carbonate radical anion, bicarbonate radical, or peroxymono-carbonate (42) that facilitate LDL oxidation.