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DYNAMICS OF FREE RADICAL FORMATION FROM THE REACTION OF PEROXIDES WITH HAEMPROTEINS AS STUDIED BY STOPPED-FLOW CHEMILUMINESCENCE

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Metal ions such as iron and copper are implicated in the generation of free radicals *in vivo*, but the dynamics of free radical production in the decomposition of hydrogen peroxide and hydroperoxides by iron complexes have not been shown unequivocally due to their rapid rates. In the present study, we have devised a stopped-flow chemiluminescence system and succeeded in elucidating the time course of the free radical formation in the decomposition of hydrogen peroxide and organic hydroperoxides by haemproteins. The rate of free radical formation was very much dependent on the metal complexes and peroxides. Methemoglobin and cytochrome c decomposed hydrogen peroxide slower than microperoxidase but generated free radicals continuously. Methyl linoleate hydroperoxide was decomposed more rapidly than hydrogen peroxide. The chemiluminescence intensity was directly proportional to the amount of hydroperoxide, and the chemiluminescence progress curve reflected the time course of free radical flux. These results show that this stopped-flow chemiluminescence system is suitable to follow the radical flux from the decomposition of peroxides by metal ions.

KEY WORDS: free radical, haemprotein, active oxygens, chemiluminescence, oxidative damage, stopped-flow analysis.

INTRODUCTION

Oxygen toxicity has been one of the most extensively studied subjects in biochemistry during the last two decades. Recent experimental, clinical and epidemiological data suggest that active oxygen species and free radicals play an important causative role in various diseases including atherosclerosis, cataract, neurological disorders, cancer and aging.[1] There is now an ample evidence which shows that the free radical-mediated oxidations of low density lipoprotein is an important initial event of atherosclerosis,[2] and the presence of iron and copper in the interior of human advanced atherosclerotic lesions has been reported.[3] The involvement of iron has been also suggested in neurodegenerative disorders.[4] Furthermore, recent reports have proposed a hypothesis that oxygen radicals may be involved in the pathway of apoptotic cell death.[5,6] It has been reported[5] that hydrogen peroxide enhances while desferrioxamine which has iron-binding and antioxidant properties protects neuronal cell death.

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It has been proposed that, under many circumstances, the production of hydroxyl radical may contribute to the pathophysiology of these conditions.[7,8] In 1894, just 100 years ago, Fenton[9] found that hydrogen peroxide, although inactive alone, promotes the oxidation of organic compounds in the presence of ferrous ion, and the combination of ferrous salts and hydrogen peroxide is called Fenton reagent.[10] Forty years later, Haber and Weiss[11] proposed that the hydroxyl radical is formed as the oxidant via the decomposition of hydrogen peroxide by ferrous ion. Despite numerous studies on this apparently simple reaction, the detailed mechanism is still controversial.[12–14]

The previous studies on Fenton reaction has centred on simple iron or its chelates, but substantially all iron *in vivo* is present as a complex with protein such as ferritin or in haemproteins. The possible role of haemproteins as a Fenton reagent has been argued.[15–17] Recently, the effect of haemoglobin and hematin on neutrophil inactivation in superoxide generating systems has been reported.[18] It is quite probable that metal complexes and peroxides play a vital role in the initiation of oxidative damage *in vivo*. The precise elucidation of the dynamics of free radical formation in metal catalyzed decomposition of peroxide is important to an understanding of their role and action. It is also important and essential in the *in vitro* kinetic study to quantify the radical flux. In the present work, we have tried and succeeded in elucidating the time course of the formation of free radicals in the decomposition of hydrogen peroxide and organic hydroperoxides by biologically relevant iron complexes by using a stopped-flow chemiluminescence technique.

MATERIALS AND METHODS

Chemicals

Microperoxidase, methemoglobin, cytochrome c and isoluminol were purchased from Sigma Chem. Co. (St. Louis, MO) and used as received. Commercial hydrogen peroxide was used without further purification. Commercial *tert*-butyl hydroperoxide was distilled under reduced pressure before use, 35°C/15 torr. The concentrations of hydrogen peroxide and *tert*-butyl hydroperoxide were measured by iodometric titration. Methyl linoleate hydroperoxide was prepared by a spontaneous autoxidation of methyl linoleate followed by purification with a silica-gel column.[19] Its concentration was calculated from the absorption at 234 nm ($\varepsilon = 28{,}000\ M^{-1}cm^{-1}$).[20]

METHODS

The schematic diagram of the analytical system used in this study is shown in Figure 1. This is based on the high performance liquid chromatography (HPLC)-chemiluminescence LC-900 system obtained from JASCO (Tokyo, Japan). The chemiluminescence intensity was measured in 2 different systems: one conventional flow system and the other a stopped-flow system. In the flow system, 20 μl sample containing an appropriate amount of hydrogen peroxide or organic hydroperoxide was injected into a flow of eluent A (water, flow rate = 1.0 ml/min), which was mixed with eluent B (flow rate = 1.5 ml/min) a sodium borate (50 mM) buffer (pH 10.0) containing the iron complex and isoluminol. For the analysis of methyl linoleate hydroperoxide, methanol and borate buffer (pH 10.0) containing 50% methanol were used as eluent A and B, respectively. A rapid mixing of peroxide and iron was attained by a special

T-joint mixer. The mixed solution follows the solid line in Figure 1. The cell was swirl-shaped (tube inner and outer diameter being 0.5 and 1.5 mm and total length 800 mm, respectively) and had a volume of 0.157 ml. In a stopped-flow system, the flow was stopped by a 6-way valve after the sample went into a cell so that the time course of chemiluminescence emission could be followed. When the flow is stopped, the eluent flows thereafter along the broken line in Figure 1. This simple apparatus made it possible to follow the radical flux from the fast decomposition of peroxides by iron complexes without conventional stopped flow apparatus which is quite expensive.

RESULTS

Figure 2 shows, as an example, how chemiluminescence emission was observed in the decomposition of hydrogen peroxide by microperoxidase with both flow and stopped-flow systems. Only transient chemiluminescence was observed in a flow system between 3 and 13 sec after injection (Figure 2, panel A). The area on the chart increased with increasing concentration of hydrogen peroxide. When the flow was stopped at 6.0 sec after injection by use of a six-way valve and the reaction mixture was kept in a cell, the chemiluminescence intensity increased at first and then decreased with time (Figure 2, panel B). This reflects the time course for formation of free radicals in the decomposition of hydrogen peroxide by microperoxidase. The area, which corresponds to the total chemiluminescence, was larger than that in panel A and increased with increasing hydrogen peroxide.

It was found that the chemiluminescence emission profile depended on the type of

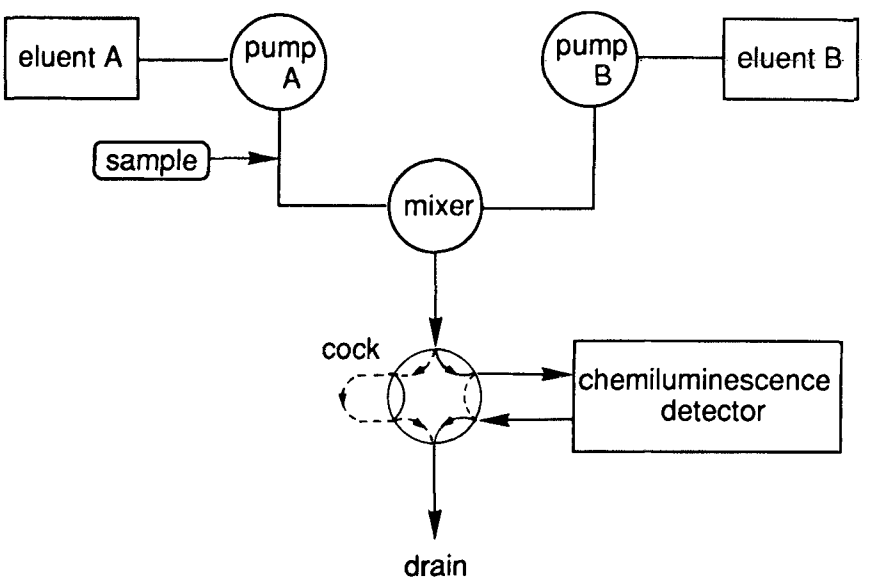

FIGURE 1 Schematic diagram of the stopped-flow chemiluminescence system. In the flow system, the mixed solution goes through the line indicated by a solid line, while in the stopped-flow system, the flow is stopped by a 6-way valve after the sample enters in the detector cell so that the time course of chemiluminescence emission, that is, free radical formation, can be followed. Under such circumstances, the eluent flows along a broken line. (See Materials and Methods)

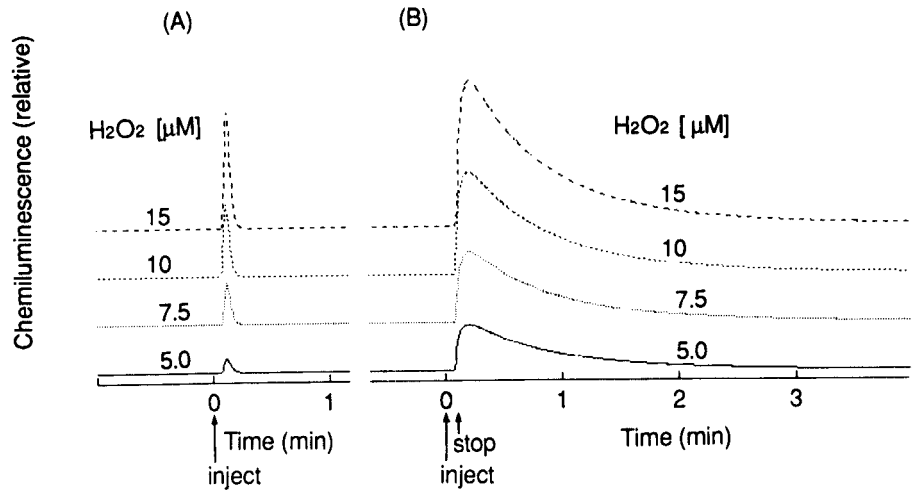

FIGURE 2 The time course profile of chemiluminescence emission in the decomposition of hydrogen peroxide by microperoxidase. The left panel (A) shows a chemiluminescence in the flow system. Twenty μl of hydrogen peroxide solution (5–15 μM) was injected into the flow of water (1.0 ml/min) and mixed with another solution of 50 mM borate buffer (pH 10.0) containing 5 mg/ml (2.5 μM assuming a molecular weight of 2000) microperoxidase and 1 mM isoluminol. The right panel (B) shows the change in the chemiluminescence intensity when the flow was stopped and the reaction mixture was held in the cell. The chemiluminescence intensity reflects the formation of free radicals in the cell. (see Materials and Methods)

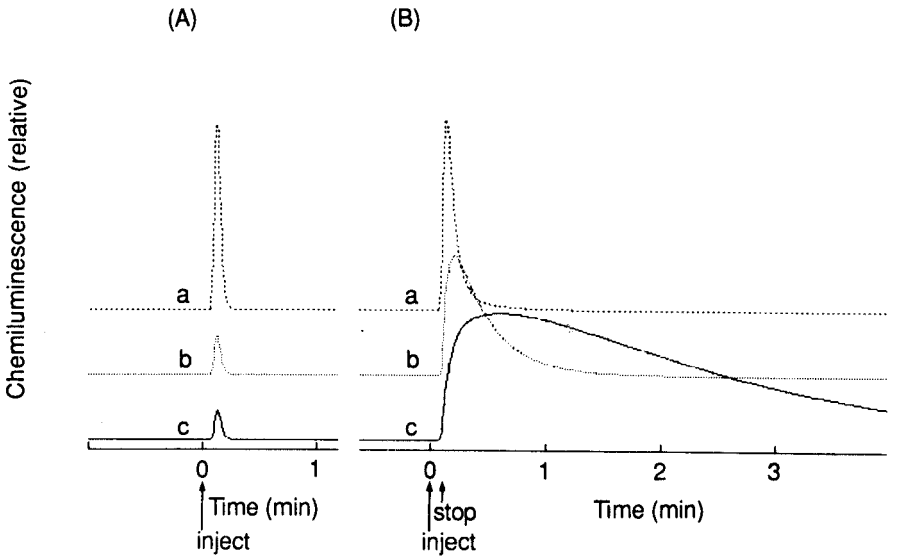

FIGURE 3 Peroxide-dependent chemiluminescence in the reaction with microperoxidase by (A) flow and (B) stopped-flow systems. Methanol and methanol/water (1/1 by vol) solution containing 50 mM sodium borate (pH 10.0), 5 mg/l (2.5 μM, see above) microperoxidase and 1 mM isoluminol were used as eluent A and B respectively. a: Methyl linoleate hydroperoxide (50 pmole); b: hydrogen peroxide (300 pmole); c: *tert*-butyl hydroperoxide (300 pmole).

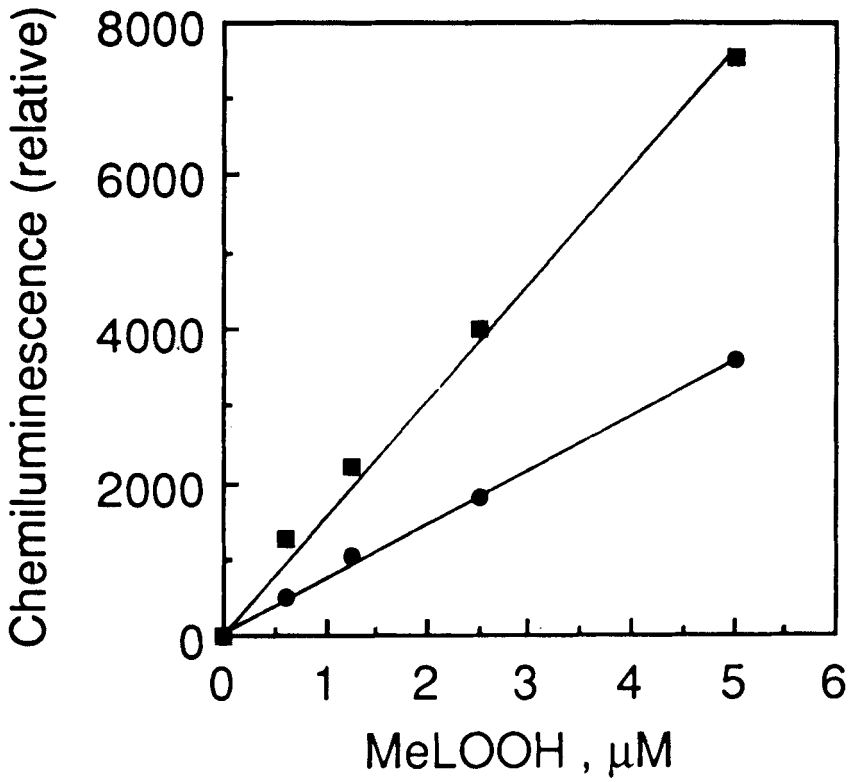

FIGURE 4 Plots of total chemiluminescence intensity as a function of methyl linoleate hydroperoxide (MeLOOH) observed in the reaction with microperoxidase (2.5 μM, see above) by flow (●) and stopped flow (■) systems. Eluent A and B were the same as in Fig. 3.

peroxide (Figure 3). In the flow system, methyl linoleate hydroperoxide gave the highest chemiluminescence intensity. Hydrogen peroxide gave stronger chemiluminescence than *tert*-butyl hydroperoxide (Figure 3-A). The chemiluminescence intensity was proportional to the peroxide concentration (Figure 4). Figure 3-B shows that the chemiluminescence intensity from methyl linoleate hydroperoxide declined rapidly, less rapidly from hydrogen peroxide but slowly from *tert*-butyl hydroperoxide. The total chemiluminescence counts measured from the area in Figure 3B were directly proportional to the concentration of peroxide as shown in Figure 4 for methyl linoleate hydroperoxide.

It was also found that the chemiluminescence intensity and time course profile depended on the type of iron complexes. Figure 5 shows the chemiluminescence observed in the decomposition of hydrogen peroxide by methemoglobin and cytochrome c. Both methemoglobin and cytochrome c gave chemiluminescence for a longer time than microperoxidase (Figure 2). In Figure 6 is shown the chemiluminescence observed in the decomposition of hydroperoxide by 3 iron complexes under the same conditions. It clearly shows that microperoxidase gave the strongest chemiluminescence, whereas those by methemoglobin and cytochrome c were undetectable under these conditions.

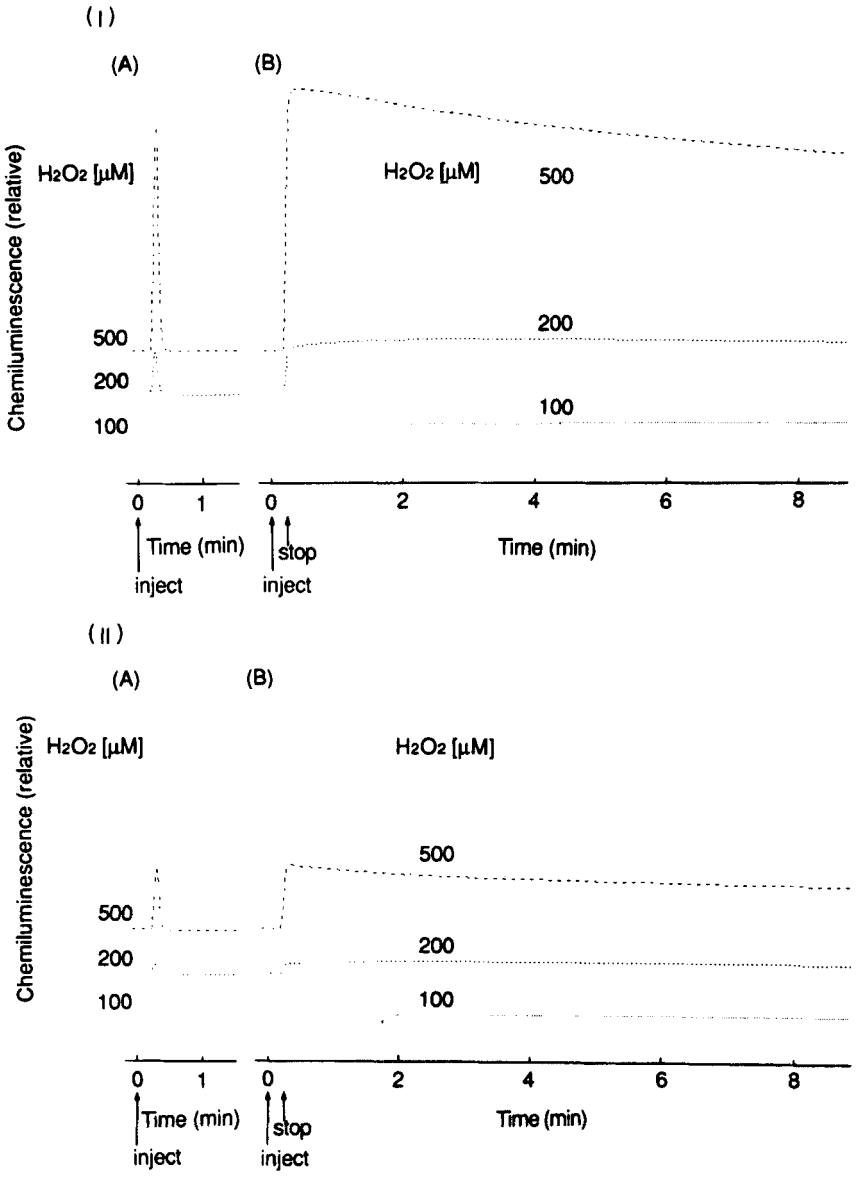

FIGURE 5 Chemiluminescence observed in the decomposition of hydrogen peroxide by (I) methemoglobin and (II) cytochrome c in (A) flow and (B) stopped-flow system. Hydrogen peroxide solution was injected into a flow of water, which was mixed with 50 mM borate buffer (pH 10.0) containing either (I) 2.5 μM methemoglobin or (II) 2.5 μM cytochrome c and 1 mM isoluminol and the chemiluminescence emission was measured as described in Materials and Methods.

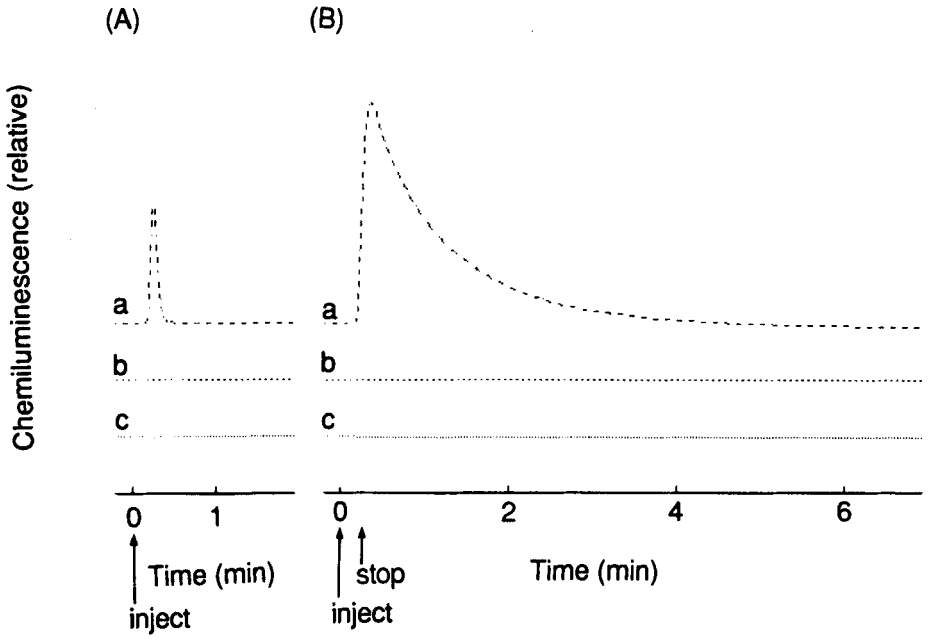

FIGURE 6 Chemiluminescence intensity observed in the decomposition of 10 μM hydrogen peroxide by 2.5 μM either (I) microperoxidase, (II) methemoglobin or (III) cytochrome c in (A) flow and (B) stopped-flow system.

DISCUSSION

The above results show that strong chemiluminescence is emitted in the decomposition of hydrogen peroxide and hydroperoxides by iron complexes in the presence of isoluminol. It was previously reported that the decomposition of hydroperoxide by cytochrome c in the presence of luminol gave chemiluminescence.[21] Interestingly, the chemiluminescence intensity and time course profile were dependent on both peroxides and iron complex (Figures 2,3,5 and 6).

It is now accepted that chemiluminescence arose from isoluminol by a following scheme,[22]

$$LH^- + \text{radical } X\bullet \rightarrow L^{\bullet -} + XH \quad (1)$$

$$L^{\bullet -} + O_2 \rightarrow L + O_2^{\bullet -} \quad (2)$$

$$L^{\bullet -} + O_2^{\bullet -} \rightarrow \text{endoperoxide} \rightarrow \text{light} \quad (3)$$

where LH^- and $L^{\bullet -}$ are isoluminol anion and isoluminol semiquinone radical respectively. It was confirmed that, when any one of the components, isoluminol, peroxide or iron complex, was omitted, little chemiluminescence was observed. Furthermore, as expected, a radical-scavenging antioxidant such as 2-carboxyl-2,5,7,8-tetramethyl-6-chromanol (Trolox), a water-soluble vitamin E analogue, and superoxide dismutase (SOD) suppressed the chemiluminescence (data not shown). Trolox scavenges oxygen radicals rapidly and inhibits the reaction 1 and SOD scavenges superoxide and inhibits the reaction 3. It was also found in the present study that a chemiluminescence with a

constant intensity was emitted when a water-soluble radical initiator, 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH), was incubated in place of metal and hydroperoxide in PBS in the presence of isoluminol under the same conditions as in Figure 2 as reported by Lissi *et al.*[23] The chemiluminescence intensity was directly proportional to the concentration of AAPH (data not shown).

These results show that the oxygen radicals generated from AAPH or metal-dependent decomposition of hydrogen peroxide or hydroperoxides attack luminol or isoluminol to eventually emit light and that the chemiluminescence intensity and time course profile reflect the amount and rate of radical flux. The chemiluminescence intensity is determined by a rate of generation of free radicals that can attack isoluminol, that is, the rate of decomposition of hydroperoxide by iron complex and the fraction of the radicals that attack isoluminol. The chemiluminescence progress curve observed in the stopped-flow system reflects the time course of radical flux. It is not clear at present what is the active radical that attacks isoluminol. It may be free hydroxyl, alkoxyl or peroxyl radical. We have previously observed that haem irons such as hemoglobin and methemoglobin initiate the oxidations of lipids and lipoproteins in the presence of hydroperoxide[24] and it is accepted that the decomposition of hydrogen peroxide and/or hydroperoxides by metal complexes is the essential initial event for lipid peroxidation.[25–29] It has been reported that the reaction of methemoglobin with hydrogen peroxide gives an iron (IV)-oxo species and the second oxidizing equivalent which is rapidly transferred to the surrounding globin generating a protein radical,[30,31] implying that not all radicals formed from peroxides are free. In any event, the chemiluminescence from isoluminol observed in the decomposition of peroxides by iron complexes show the formation of free active oxygen species capable of attacking substrates such as lipids and proteins and its time course profile observed in the present stopped-flow chemiluminescence system reflects the change in flux of such active oxygen species with time.

The present study shows that methemoglobin and cytochrome c decompose peroxides relatively slowly and generate active oxygen species continuously, whereas microperoxidase decomposes peroxides rapidly. It may be noteworthy that smaller molecule such as ferrous chloride decomposes hydrogen peroxide more rapidly than microperoxidase (data not shown). These points should be taken into consideration in the *in vitro* oxidation study induced by iron.

The chemiluminescence has been applied to the measurement of hydroperoxide in biological samples such as plasma.[32,33] The above results show that the relative chemiluminescence intensity, that is, analytical sensitivity, depends markedly on the type of hydroperoxide and iron complex, and also on the analytical conditions such as dead volume in a flow system and flow rate of the eluent.

In conclusion, the present study shows that the stopped-flow chemiluminescence system is convenient to follow the flux of active oxygen species capable of causing oxidative damage in the decomposition of peroxides by iron complexes.

References

1. H. Sies, ed., (1991) Oxidative Stress: Oxidants and Antioxidants. Academic Press, London.
2. D. Steinberg (1991) Antioxidants and atherosclerosis. *Circulation*, **84,** 1420–1425.
3. C. Smith, M.J. Mitchinson, O.I. Aruoma and B. Halliwell (1992) Stimulation of lipid peroxidation and hydroxyl radical generation by the contents of human atherosclerotic lesions. *Biochemical Journal*, **286,** 901–905.
4. B. Halliwell (1989) Oxidants and the central nervous system: some fundamental questions. *Acta Neurological Scandinavia*, **126,** 23–33.

5. D.J. Kane, T.A. Sarafian, R. Anton, H. Hahn, E.B. Gralla, J.S. Valentine, T. Ord and D.E. Bredesen (1993) Bcl-2 inhibition of neural death: decreased generation of reactive oxygen species. *Science*, **262,** 1274–1277.
6. T.A. Sarafian and D.E. Bredesen (1994) Is apoptosis mediated by reactive oxygen species? *Free Radical Research*, **21,** 1–8.
7. B. Halliwell and J.M.C. Gutteridge (1985) The importance of free radicals and catalytic metal ions in human diseases. *Molecular Aspects of Medicine*, **8,** 89–193.
8. C. von Sonntag and H-P. Schuchmann (1994) Suppression of hydroxyl radical reactions in biological systems. In *Methods in Enzymology* (ed. L. Packer), Academic Press, San Diego, pp. 47–56.
9. H.J.H. Fenton (1894) Oxidation of tartaric acid in the presence of iron. *Journal of the Chemical Society*, **65,** 899–910 (1894).
10. W.H. Koppenol (1993) The centennial of the Fenton reaction. *Free Radical Biology & Medicine*, **15,** 645–651.
11. F. Haber and J.J. Weiss (1934) The catalytic decomposition of H_2O_2 by iron salts. *Proceedings of the Royal Society, London*, **A147,** 332–351.
12. S. Goldstein, D. Meyerstein and G. Czapski (1993) The Fenton reagents. *Free Radical Biology & Medicine*, **15,** 435–445.
13. K.M. Schaich (1992) Metals and lipid oxidation. Contemporary issues. *Lipids*, **27,** 209–218.
14. H.C. Sutton and C.C. Winterbourn (1989) On the participation of higher oxidation states of iron and copper in Fenton reactions. *Free Radical Biology & Medicine*, **6,** 53–60.
15. S.M.H. Sadrzadeh, E. Graf, S.S. Panter, P.E. Halleway and J.W. Eaton (1984) Hemoglobin. A biologic Fenton reagent. *Journal of Biological Chemistry*, **259,** 14354–14356.
16. A. Puppo and B. Halliwell (1988) Formation of hydroxyl radicals from hydrogen peroxide in the presence of iron. *Biochemical Journal*, **249,** 185–190.
17. C. Rice-Evans (1993) Oxidized low density lipoproteins. in Free Radicals: From Basic Science to Medicine (G. Poli, E. Albano, M.U. Dianzani, eds.) Basel: Birkhauser, pp. 323–339.
18. Y.-M. Kim, I. Yamazaki and L.H. Piette (1994) The effect of hemoglobin, hematin, and iron on neutrophil inactivation in superoxide generating systems. *Archives of Biochemistry and Biophysics*, **309,** 308–314.
19. Y. Yamamoto, N. Saeki, H. Haga, E. Niki and Y. Kamiya (1984) Oxidation of lipids. IX. Decomposition of methyl linoleate and methyl linolenate hydroperoxides in solution. *Bulletin of Chemical Society of Japan*, **57,** 3177–3181.
20. H.W.-S. Chan and G. Levett (1977) Autoxidation of methyl linoleate. Separation and analysis of isomeric mixtures of methyl linoleate hydroperoxides and methyl hydroxylinoleates. *Lipids*, **12,** 99–104.
21. T. Iwaoka and F. Tabata (1984) Chemiluminescent assay of lipid peroxide in plasma using cytochrome c heme peptide. *FEBS Letters*, **178,** 47–50.
22. G. Merenyi and J.S. Lind (1980) Role of a peroxide intermediate in the chemiluminescence of luminol. A mechanistic study. *Journal of American Chemical Society*, **102,** 5830–5835.
23. E. Lissi, C. Pascual and M.D. Del Castillo (1992) Luminol luminescence induced by 2,2′-azo-bis (2-amidinopropane) thermolysis. *Free Radical Research Communications*, **17,** 299–311.
24. Y. Yoshida, K. Kashiba and E. Niki (1994) Free radical-mediated oxidation of lipids induced by hemoglobin in aqueous dispersions. *Biochimica et Biophysica Acta*, **1201,** 165–172.
25. C.E. Thomas and R.L. Jackson (1991) Lipid hydroperoxide involvement in copper-dependent and independent oxidation of low density lipoproteins. *The Journal of Pharmacology and Experimental Therapeutics*, **256,** 1182–1188.
26. V.J. O'Leary, V.M. Darley-Usmar, L.J. Russell and D. Stone (1992) Pro-oxidant effects of lipoxygenase-derived peroxides on the copper-initiated oxidation of low-density lipoprotein. *Biochemical Journal*, **282,** 631–634.
27. G. Dee, C. Rice-Evans, S. Obeyesekera, S. Meraji, M. Jacobs and K.R. Bruckdorfer (1991) The modulation of ferryl myoglobin formation and its oxidative effects on low density lipoprotein by nitric oxide. *FEBS Letters*, **294,** 38–42.
28. B. Frei and J.M. Gaziano (1993) Content of antioxidants, preformed lipid hydroperoxides and cholesterol as predictors of the susceptibility of human LDL to metal ion dependent and independent oxidation. *Journal of Lipid Research*, **34,** 2135–2145.
29. N. Noguchi, Y. Yoshida, H. Kaneda, Y. Yamamoto and E. Niki (1992) Action of ebselen as an antioxidant against lipid peroxidation. *Biochemical Pharmacology*, **44,** 39–44.
30. M.J. Davies, B. Gilbert and R.M. Haywood (1991) Radical-induced damage to proteins: E.S.R. spin-trapping studies. *Free Radical Research Communications*, **15,** 111–127.
31. K.M. McArthur and M.J. Davies (1993) Detection and reactions of the globin radical in haemoglobin. *Biochimica et Biophysica Acta*, **1202,** 173–181.
32. Y. Yamamoto (1994) Chemiluminescence-based high-performance liquid chromatography assay of

lipid hydroperoxides. In Methods in Enzymology, Vol. 233 (L. Packer, ed.) Academic Press, London, pp. 319–324.
33. T. Miyazawa, K. Fujimoto, T. Suzuki and K. Yasuda (1994) Determination of phospholipid hydroperoxides using luminol chemiluminescence-high-performance liquid chromatography. In Methods in Enzymology, Vol. 233 (L. Packer, ed.) Academic Press, London, pp. 324–332.

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