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Luminol Chemiluminescence and Peroxidation of Unsaturated Fatty Acid Induced by the Xanthine Oxidase System: Effect of Oxygen Radical Scavengers[1)]

TOSHIAKI MIURA* and TAKETO OGISO

Hokkaido Institute of Pharmaceutical Sciences, 7–1 Katsuraoka-cho, Otaru 047–02, Japan

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Addition of luminol to the xanthine oxidase system caused the production of chemiluminescence (CL). Superoxide dismutase (SOD) and singlet oxygen ($^1O_2$) scavengers inhibited the CL, whereas catalase and hydroxyl radical (HO·) scavengers had no significant effect, suggesting that $^1O_2$ species derived from O_2^- is responsible for the light emission of luminol. Addition of unsaturated acids to the xanthine oxidase system in the presence of luminol resulted in quenching of CL, and peroxidation of the unsaturated fatty acids was observed. SOD and $^1O_2$ scavengers also inhibited the peroxidation of arachidonate, indicating that $^1O_2$ generated in the xanthine oxidase system participates in the peroxidation reaction.

Keywords——chemiluminescence; luminol; xanthine oxidase; superoxide dismutase; singlet oxygen scavenger; unsaturated fatty acid; lipid peroxidation

We have previously reported that superoxide dismutase (SOD, EC 1.15.1.1) and singlet oxygen ($^1O_2$) scavengers strongly inhibited the lipid peroxidation of erythrocyte membranes and phosphatidylcholine liposomes induced by incubation in the xanthine oxidase superoxide (O_2^-) generating system.[2)] These results suggested that $^1O_2$ derived from O_2^- is responsible for initiating the peroxidation reaction. A relatively long-lived electronically excited species of oxygen, $^1O_2$ is known to be produced in photosensitized and biochemical reactions.[3)] However, there has not yet been a direct demonstration of $^1O_2$ being produced in the xanthine oxidase system. In the present study, we monitored the production of $^1O_2$ in the xanthine oxidase system by using luminol-dependent chemiluminescence (CL) and organic substances known to be chemical scavengers of $^1O_2$. In addition, we investigated the participation of $^1O_2$ in lipid peroxidation by measuring the CL quenching by unsaturated fatty acids.

Experimental

Materials——Xanthine oxidase (from butter milk), obtained from Boehringer Mannheim GmbH, Germany, was dialyzed against 10 mM phosphate buffer, pH 7.4 containing 0.15 M NaCl before use. SOD (from bovine blood) and catalase (from bovine liver, thymol-free) were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. Lyophilized powder of catalase was further purified as described previously.[2)] Arachidonic acid was obtained from PL Biochemicals, Inc., Milwaukee, Wis., U.S.A., while caprylic, linoleic and linolenic acid were from Kanto Chemical Co., Inc. Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), histidine, 1,4-diazabicyclo-(2,2,2)-octane (DABCO), dimethylfuran, mannitol, sodium benzoate and sodium azide were obtained from Wako Pure Chemical Industry Co., Ltd., while thiobarbituric acid (TBA) was from Merck Japan Ltd. TBA was recrystallized three times from water before use. Malondialdehyde (MDA) was prepared by hydrolysis of 1,1,3,3-tetra-ethoxypropane, which was obtained from Tokyo Kasei Kogyo Co., Ltd. All other chemicals used were of the highest purity commercially available.

Reaction System and Chemiluminescence——The xanthine oxidase system contained 5 mM hypoxanthine, 0.15 M NaCl and 0.025 unit of xanthine oxidase in 3.0 ml of 10 mM phosphate buffer, pH 7.4. To remove trace metals, the buffer solutions used in all experiments were treated with Chelex-100 resin. Activity of xanthine oxidase was assayed

at 25 °C by measuring the absorption of uric acid at 293 nm. One unit of activity was defined as 1.0 μmol of hypoxanthine converted to uric acid per min. The O_2^- production in the reaction system was monitored spectrophotometrically by measuring the rate of reduction of nitro blue tetrazolium and ferricytochrome c.[4] CL was produced in the glass counting vials by the addition of luminol (10.0 μM) to the xanthine oxidase system in the dark at room temperature (25 °C). CL was measured as counts per min with a Packard model 3385 liquid scintillation counter operated in the out-of-coincidence mode using the narrow tritium iso-set module as described by Videla.[5] The light intensity was represented as highest peak signal (cpm) minus the background level during the course of the reaction. The incubation mixture used for the determination of deuterium effect was prepared by replacing most of the H_2O in the incubation mixture with D_2O (more than 90%). Other additions and omissions of reagents were as specified in the figure and tables.

Quenching of CL by Fatty Acids and Lipid Peroxidation——Fatty acids were dispersed in the xanthine oxidase system containing 0.02% Lubrol, followed by the addition of 10 μM luminol. Quenching activity of fatty acids was determined by measuring the light intensity in the presence and absence of fatty acids as described by Krasnovsky *et al.*[6] Lipid peroxidations were measured in terms of MDA formation as described previously.[2] When dimethylfuran was used as a scavenger, MDA formation could be detected by replacement of phosphoric acid with trichloracetic acid. To prevent further peroxidation of fatty acids during the assay procedure, 0.1 ml of 1% butylated hydroxytoluene in dimethylsulfoxide was added to the TBA reagent.

Results

Chemiluminescence of Luminol in the Xanthine Oxidase System

The addition of luminol to the xanthine oxidase system caused production of CL as shown in Fig. 1. The light intensity reached a maximum level immediately after the initiation of the reaction, depending on the luminol concentrations, and then gradually decreased to the background level. No significant CL was detected under our experimental conditions in the absence of luminol or in an atmosphere of N_2 gas. The insert shows that the highest peak signal recorded during the course of the reaction was proportional to xanthine oxidase concentration. The addition of luminol to the xanthine oxidase system did not affect the rate

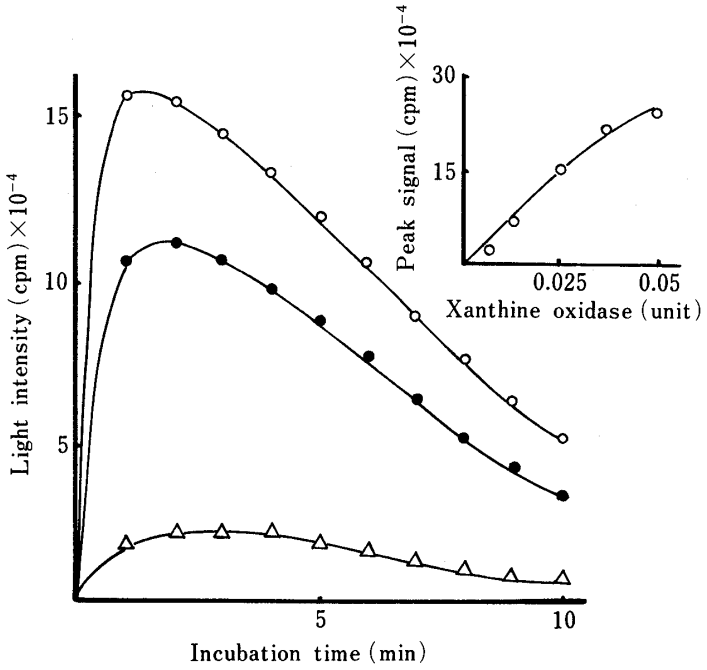

Fig. 1. Chemiluminescence in the Xanthine Oxidase System

The xanthine oxidase system contained 0.15 M NaCl, 5 mM hypoxanthine and 0.025 unit of xanthine oxidase in 3.0 ml of 10 mM phosphate buffer, pH 7.4. Various amounts of luminol (—○—, 10 μM; —●—, 5 μM; —△—, 1 μM) were added. Light intensity was measured by the method described in Experimental. The insert shows the highest peak signal from the reaction system containing various amounts of xanthine oxidase in the presence of 10 μM luminol.

of O_2^- production.

As shown in Table I, the addition of native SOD to the reaction system almost completely inhibited CL, whereas denatured SOD was without effect. On the other hand, catalase had a negligible effect on CL even at concentrations up to 10 μg/ml. The addition of both SOD and catalase inhibited the light emission of luminol to nearly the same extent as that of SOD alone (data not shown). No CL was produced by addition of 1.0 mM H_2O_2 to the xanthine oxidase system in the absence of hypoxanthine. These results indicate that O_2^- rather than H_2O_2 is responsible for the light emission of luminol in the xanthine oxidase system.

Effect of Some Radical Scavengers

As shown in Table II, DABCO, which is an efficient quencher of $^1O_2$, completely inhibited the CL of luminol. Other quenchers of $^1O_2$, histidine, dimethylfuran and sodium azide also inhibited more than 70% of the light emission of luminol. Sodium benzoate and mannitol, which are known to react with hydroxyl radical (HO·), had no significant effect, indicating that the emission of luminol CL was independent of HO·. The rate of substrate oxidation by xanthine oxidase and concomitant generation of O_2^- were little affected by addition of the chemical scavengers used. These results strongly suggest that $^1O_2$ species derived from O_2^- is responsible for the light emission of luminol.

Quenching Activity and Peroxidation of Unsaturated Fatty Acids

To further examine the possible generation of $^1O_2$ and the reactivity of $^1O_2$ with biological membrane lipids, the quenching activity and peroxidation of fatty acids were determined in the xanthine oxidase system. As shown in Table III, the addition of unsaturated

TABLE I. Effects of SOD and Catalase on Chemiluminescence

Addition	Concentration (μg/ml)	Light intensity (cpm)	Relative intensity (%)
None	—	157195 ± 6565	100
SOD	0.01	69558 ± 4337	44.2
	0.10	29640 ± 5245	18.9
	1.0	2537 ± 698	1.6
Denatured SOD	1.0	143451 ± 5567	91.2
Catalase	1.0	150485 ± 2899	95.7
	10.0	136713 ± 7943	86.5

Various amounts of SOD and catalase were added to the xanthine oxidase system before initiation of the reaction. Light intensity was measured immediately after the addition of luminol to the reaction system. Each value represents the mean ± S.E. of triplicate experiments.

TABLE II. Effect of Oxygen Radical Scavengers on Chemiluminescence

Scavenger	Concentration (mM)	Light intensity (cpm)	Relative intensity (%)
None	—	157195 ± 6565	100
DABCO	10	5344 ± 894	3.4
Histidine	10	46035 ± 7575	29.3
Dimethylfuran	1.0	45258 ± 5651	28.8
Sodium azide	10	42618 ± 5019	27.1
Sodium benzoate	10	152391 ± 16048	96.9
Mannitol	10	121694 ± 1756	77.4

Various scavengers were added to the xanthine oxidase system before initiation of the reaction. Each value represents the mean ± S.E. of triplicate experiments.

Table III. Quenching Activity and Peroxidation of Unsaturated Fatty Acids

Experimental conditions	Concentration (mM)	Light intensity (cpm)	Quenching activity (Lo/L)	MDA formation (nmol)
None	—	157195± 6565	—	—
Arachidonic acid	1.0	60119±10471	2.61	4.90
	0.5	80512±11238	1.95	3.25
Linolenic acid	1.0	134176± 8719	1.17	ND
	0.5	145449±14568	1.08	1.72
Linoleic acid	1.0	142744± 1944	1.10	ND
	0.5	148298±13162	1.06	1.45
Caprylic acid	1.0	168176±13593	0.93	0
	0.5	162303± 7659	0.97	0

Fatty acids were dispersed in the xanthine oxidase system containing 0.02% Lubrol. Light intensity was measured immediately after addition of luminol (10 μM) to the reaction system at room temperature (25 °C). Lo and L indicate the intensity of chemiluminescence in the absence and presence of the fatty acids, respectively. MDA formed was determined after incubation for 60 min at 37 °C. Values of light intensity represent the mean ± S.E. of triplicate experiments. Values of MDA formed are the mean of duplicate experiments. ND: not determined.

Table IV. Effects of SOD and Oxygen Radical Scavengers on the Peroxidation of Arachidonic Acid

Addition	Concentration	MDA formation (nmol)	Inhibition (%)
None	—	4.90	—
SOD	0.1 (μg/ml)	2.10	58.9
	1.0 (μg/ml)	0.91	81.4
Dimethylfuran	0.5 (mM)	2.95	39.8
	1.0 (mM)	1.52	69.0
Histidine	1.0 (mM)	3.10	36.7
	10.0 (mM)	2.93	40.2
Sodium azide	10.0 (mM)	3.15	35.7
DABCO	10.0 (mM)	4.00	18.3
Catalase	1.0 (μg/ml)	9.54	94.7[a]
Sodium benzoate	10.0 (mM)	5.16	5.3[a]
Mannitol	10.0 (mM)	4.63	5.5

SOD and various scavengers were added to the xanthine oxidase system containing 1.0 mM arachidonic acid and 0.02% Lubrol. After incubation for 60 min at 37 °C, MDA formed was determined as described in Experimental. Each value represents the mean of duplicate experiments. *a*) Stimulation.

fatty acids to the xanthine oxidase system resulted in quenching of CL. The order of quenching activity was arachidonic > linolenic > linoleic acid, and depends on the number of double bonds in fatty acids. During the course of incubation, lipid peroxidation was observed as indicated by an increase in the formation of MDA. Arachidonic acid was most sensitive to the peroxidation reaction. When linolenic and linoleic acid (0.5 mM) were added to the reaction system, the amounts of MDA formed were about 1.7 and 1.5 nmol, respectively. However, the addition of caprylic acid caused neither substantial inhibition of CL nor lipid peroxidation. These results probably suggest that the quenching of CL by unsaturated fatty acids is a consequence of the reaction between $^1O_2$ and double bonds in the fatty acids.

Inhibition of Arachidonic Acid Peroxidation by Various Scavengers

To determine whether $^1O_2$ is involved in the lipid peroxidation or not, the effects of oxygen radical scavengers were examined. As shown in Table IV, addition of SOD to the

xanthine oxidase system caused strong inhibition of arachidonate peroxidation. Dimethylfuran and histidine (1.0 mM) inhibited this peroxidation reaction by about 70 and 37%, respectively, while sodium azide (10 mM) inhibited it by about 36%. However, the inhibitory effect of 10 mM DABCO was only about 18%. Mannitol and benzoate showed no significant effect on the lipid peroxidation of arachidonic acid. These results indicate that $^1O_2$ generated in the xanthine oxidase system at least partially participates in the unsaturated fatty acid peroxidation reaction. Addition of catalase to the reaction system caused a large enhancement of peroxidation of arachidonic acid.

Discussion

Several attempts have been made to detect the generation of $^1O_2$ in biological systems. In general, three methods have been used: (1) the use of quenchers or traps; (2) the detection of low level CL of $^1O_2$ decay; (3) the increase of $^1O_2$ life time in D_2O. A combination of two of these methods should yield reliable evidence for the involvement of $^1O_2$ in a biological reaction. The excited $^1O_2$ returns to the ground state with emission of weak CL at 1270 nm, which is out of the range of commonly used CL detectors.[7] The instantaneous light emission at 1270 nm has been reported to be shifted into the visible region and amplified by the addition of luminol.[8] Hodgson *et al.*,[9] however, pointed out that the CL of luminol induced in the xanthine oxidase systems at pH 10.2 was due to the accumulation of H_2O_2, which in turn reacts with O_2^- to produce the reactive species of HO· (Haber–Weiss reaction). In the present study, the aerobic xanthine oxidase reaction at pH 7.4 produced the CL of luminol. However, neither catalase nor scavengers of HO· inhibited this CL, which was instead markedly inhibited by both SOD and scavengers of $^1O_2$ (Tables I and II). These results strongly suggest that both O_2^- and $^1O_2$ are essential oxygen radicals in the CL-producing process. The apparent discrepancies between our results and those of Hodgson *et al.* may be attributed to the differences in experimental conditions. Although the data are not shown, the light emission of luminol was enhanced by incubation at pH higher than 8.0, and this CL was no longer inhibited by scavengers of $^1O_2$. Moreover, the direct additon of H_2O_2 to the reaction system in the presence of luminol caused a strong emission of light at pH 8.5, whereas the CL was scarcely observed at pH 7.4. These results suggest that H_2O_2 generated in the xanthine oxidase system at pH 7.4 is unlikely to be a species responsible for the emission of luminol CL. Khan[10] showed by using a high-sensitivity spectrometer in the 1.0—1.6 μm region that spontaneous dismutation of O_2^- resulted in the formation of $^1O_2$. Other workers[11] have reported that the protonated form of O_2^- is required for the formation of $^1O_2$ according to the following scheme:

$$HO_2 + O_2^- + H^+ \longrightarrow {}^1O_2 + H_2O_2$$

$$HO_2 + HO_2 \longrightarrow {}^1O_2 + H_2O_2$$

It appears possible, therefore, that $^1O_2$ is generated in the xanthine oxidase system at physiological pH.

The reactivity of $^1O_2$ with unsaturated fatty acids and biological membranes has been demonstrated directly in the photosensitized peroxidation reaction.[12] Krasnovsky *et al.*[6] have shown by using the photosensitized luminescence of $^1O_2$ that unsaturated fatty acids quench $^1O_2$ depending on the number of double bonds. We found that the addition of unsaturated fatty acids to the xanthine oxidase system results in quenching of the luminol CL and a concomitant increase in MDA formation, while neither significant quenching activity nor MDA formation was observed with a saturated fatty acid, caprylic acid (Table III). From these results, it seems most reasonable to conclude that unsaturated fatty acids

quench $^1O_2$ *via* a chemical reaction, presumably the peroxidation reaction between $^1O_2$ and double bonds in the fatty acids. The peroxidation of arachidonic acid was inhibited by addition of either SOD or $^1O_2$ scavengers such as dimethylfuran, histidine and sodium azide (Table IV), indicating that O_2^- generated by the action of xanthine oxidase could decompose to yield $^1O_2$, which is responsible for initiating the peroxidation of arachidonic acid. However, DABCO (10 mM), another scavenger of $^1O_2$, markedly inhibited the light emission of luminol (Table II) but caused only 18% inhibition of arachidonate peroxidation. Previously, we demonstrated that the addition of DABCO to the xanthine oxidase system caused about 56% inhibition of the peroxidation of erythrocyte membranes and about 93% inhibition in the liposome system,[2] so the small inhibitory effect of DABCO on arachidonate peroxidation might be explainable on the basis of different configurations of the model membrane lipids and the detergent-dispersed arachidonic acid. Actually, lipid peroxidation is known to be affected differently by various scavenging agents when intact ghosts are compared with detergent-dispersed ghosts.[13]

Several workers have demonstrated that the peroxidation of linolenic acid and arachidonic acid induced by incubation in the xanthine oxidase system was inhibited by both SOD and catalase, suggesting possible participation of both O_2^- and H_2O_2.[14] In contrast to these results, we found no evidence for the involvement of H_2O_2 in our peroxidation reaction. In fact, the arachidonate peroxidation was not inhibited but enhanced by the addition of catalase under our experimental conditions, as discussed in the previous paper.[2] Furthermore, the inability of either mannitol or benzoate to inhibit the peroxidation reaction rules out the involvement of HO· (Table IV). The results of these experiments provide evidence that $^1O_2$ derived from O_2^- plays an important role in initiating lipid peroxidation.

The solvent deuterium isotope effect, which increases the life-time of $^1O_2$, has often been used as a simple test for the involvement of $^1O_2$ in any chemical, enzymatic or photochemical process.[15] Although the data are not shown, the substitution of D_2O for H_2O in the reaction system caused a marked inhibition of xanthine oxidase activity (about 80%); hence sufficient evidence for the participation of $^1O_2$ in the luminol CL and lipid peroxidation could not be obtained in D_2O. However, the inhibitory effects of the singlet oxygen scavengers on the luminol CL and lipid peroxidation presented here can probably be taken to imply a role of $^1O_2$ in both processes.

References and Notes

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