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Detection of Vitamin C-induced Singlet Oxygen Formation in Oxidized LDL Using MCLA as A Chemiluminescence Probe

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Abstract In this study, it was observed that addition of vitamin C (vit C) to oxidized low-density lipoprotein (Ox-LDL) by cupric ions (Cu^{2+}) could result in the formation of singlet oxygen ($^1O_2$). In experiments, $^1O_2$ was detected by chemiluminescence method using a Cypridina luciferin analog, 2-methyl-6-(*p*-methoxyphenyl)-3, 7-dihydroimidazo [1, 2-a] pyrazin-3-one (MCLA), as a selective and sensitive chemiluminescence probe. Additional experimental evidence for the formation of $^1O_2$ came from the quenching effect of sodium azide (NaN_3) on vit C-induced chemiluminescence in the reaction mixture of LDL-Cu^{2+}-MCLA. Analysis based on the experimental results demonstrated the plausible reaction mechanism is that vit C first converts Cu^{2+} to its reduced state and vit C becomes vit C radical itself, thereby stimulating the formation of peroxyl and alkoxyl radicals, and bimolecular reaction of peroxyl radicals results in $^1O_2$ production in the systems studied.

Key words vitamin C; singlet oxygen; MCLA; low-density lipoprotein; Cu^{2+}

The lowest excited singlet oxygen ($^1O_2$) plays an important role in biological systems[1-3]. The detection of near-infrared spectrometry at 1.27 μm can give precise evidence for the existence of $^1O_2$. However, despite the advances in $^1O_2$ detection using near-infrared spectrometry employing sensitive semiconductor-based detectors, the identification and direct observation of the highly reactive short-lived $^1O_2$ in biological system remains extremely difficult because the quantum yield of its infrared emission is 10^{-6} at best.

Uehara *et al*.[3,4] detected and quantified small amounts of $^1O_2$ generated in a myeloperoxidase-H_2O_2-halide ion system and heme-catalyzed decomposition of linoleic acid hydroperoxide using *Cypridina luciferin analogs*, 2-methyl-6-phenyl-3, 7-dihydroimidazo [1, 2-a] pyrazin-3-one (CLA), and 2-methyl-6-(*p*-methoxyphenyl)-3, 7-dihydroimidazo [1, 2-a] pyrazin-3-one (MCLA) as chemiluminescence probes in the presence of superoxide dismutase (SOD). We also found that MCLA was a very useful and sensitive chemiluminescence probe for the identification of both superoxide anion ($O_2^{\cdot-}$) and $^1O_2$ generated in biological systems. In this study, the generation of $^1O_2$ induced by addition of vitamin C (vit C) to oxidized low-density lipoprotein (LDL) systems was detected by using MCLA. The mechanism of reaction between MCLA with $^1O_2$ or $O_2^{\cdot-}$ probably is producing a dioxetane analog which decarboxylates and protonates to an excited carbonyl compound which deexcites to emit light at 465 nm[5].

Vit C is a major water-soluble antioxidant in human plasma[6]. The use of vit C for antioxidant therapy has been advocated because of its ability to scavenge reactive oxygen species (ROS), which can damage cellular macromolecules such as DNA and proteins. However, the potential of vit C for prooxidant activity in the presence of transition metal ions has also been recognized[7]. Ultraweak luminescence induced by vit C in Characeae cells had been studied by Jaskowska and coworkers[8]. In this experiment, using MCLA we observed chemiluminescence emission when vit C was added to LDL preoxidized by cupric ions (Cu^{2+})[9]. Based on the selectivity of MCLA-mediated chemiluminescence to $O_2^{\cdot-}$ and $^1O_2$ and the effects of various quenchers, it appeared that the observed light emission resulted from $^1O_2$ formation. The results showed that together

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with Cu^{2+}, vit C probably could decompose lipid hydroperoxides by one-electron transfer reaction and this activity might be linked to the generation of $^1O_2$.

1 Materials and Methods

1.1 Materials

MCLA, purchased from Tokyo Kasei Kogyo Co. Ltd., was dissolved in double-distilled water and stored at - 20 ℃ until needed. MCLA concentrations were based upon $\varepsilon_{430\ nm} = 9.6 \times 10^3\ (mol/L)^{-1} \cdot cm^{-1}$. Cu-Zn superoxide dismutase (SOD, from bovine erythrocytes) was obtained from the Sigma Chemical Co. L-ascorbic acid (vit C), mannitol, sodium azide (NaN_3), cupric sulphate 5-hydrate ($CuSO_4 \cdot 5H_2O$), ethylenediaminetetracetic acid (EDTA) and other chemicals were all of AR grade and made in China.

LDL was separated from the healthy human serum by one-step ultracentrifugation in discontinuous gradient according to the procedure described by Zhang *et al*[10]. The concentration of protein was determined by the modified Lowry procedure described by Markwell *et al*[11]. In order to prevent oxidation of LDL, all stock solutions were bubbled with pure N_2 before use.

1.2 Methods

Components of the reaction mixture were prepared just before using. The standard reaction mixture contained LDL (200 mg/L), Cu^{2+} (5 μmol/L), MCLA (2 μmol/L), vit C (1 mmol/L) and 10 mmol/L phosphate buffer saline (PBS) at pH 7.2 in a total volume of 1.5 ml. The chemiluminescence reactions were initiated by rapid injection of 20 μL of vit C solution to the preoxidized LDL mixture contained 5 μmol/L Cu^{2+} and 2 μmol/L MCLA. As contrast, the chemiluminescence also were detected when vit C was added to MCLA plus LDL pretreated with 0.2 mmol/L EDTA to chelate trace transition metals but not preincubated with Cu^{2+}, and when vit C was added to MCLA plus Cu^{2+} in the absence of LDL.

For confirming the origin of MCLA-mediated chemiluminescence, the peak light intensity change was recorded after the addition of various quenchers of reactive species to the LDL-Cu^{2+}-vit C-MCLA reaction system. The quenchers used in experiments included 1 μmol/L SOD, 1 mmol/L NaN_3 and 10 mmol/L mannitol.

In order to make sure the relationship between the MCLA-mediated chemiluminescence intensity and the concentration of conjugated diene (CD), lipid peroxidation of LDL was modulated using different incubation times (0 h, 2 h, 4 h, 6 h and 8 h) at 37 ℃ and then chemiluminescence and CD experiments were performed at intervals of 2 h for a period of 8 h. Measurement of CD was performed according to the literature[12] by recording the absorbance at 234 nm of the incubation suspension of Cu^{2+} and LDL in 0.01 mol/L PBS at pH 7.2.

All chemiluminescence measurements were carried out at 25 ℃ by using a highly sensitive Intensified Charge-Coupled Device (ICCD, model: ICCD-576-s/1) detector (from Princeton Instrument Inc. USA), which was cooled to - 40 ℃ by a controller. Samples in polystyrene tubes were placed in a light-tight box. Through a photographic lens (Nikon 50 mm, f 1.4) the chemiluminescence intensity values were collected and recorded in appropriate exposure time, then the results of chemiluminescence measurement were displayed and processed with a Winview software in a computer.

2 Results

2.1 MCLA-mediated chemiluminescence in LDL-Cu^{2+}-vit C systems

MCLA can selectively react with both $O_2^{\cdot-}$ and $^1O_2$ generated in biological systems and $O_2^{\cdot-}$ can be eliminated by SOD at catalytic amounts. In experiments, we observed that MCLA showed appreciable spontaneous light emission by itself in the system which vit C was omitted from the reaction solution. However, the luminescence could be inhibited partially by addition of 0.2 mmol/L EDTA and it was comparatively stable, and its level was taken as a background.

It has been confirmed that Cu^{2+} can initiate the lipid peroxidation of LDL[13, 14]. When Vit C was added to a solution of MCLA plus LDL exposed to Cu^{2+}, MCLA-mediated chemiluminescence appeared, reached the maximum and decreased rapidly (Fig. 1).

In contrast, no increase of chemiluminescence intensity was observed when LDL was pretreated

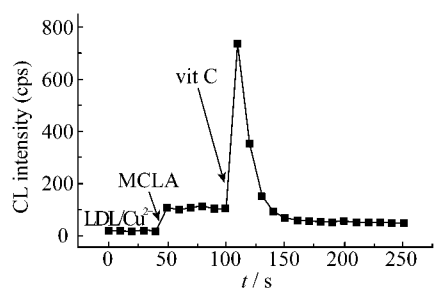

Fig. 1 MCLA-mediated chemiluminescence in LDL-Cu^{2+}-vit C system in 0.01 mol/L PBS of pH 7.2

LDL (200 mg/L) preoxidized for 2 h with Cu^{2+} (5 μmol/L) at 37 ℃. MCLA (2 μmol/L) and vit C (1 mmol/L) were added at the point indicated by the arrow. CL, chemiluminescence.

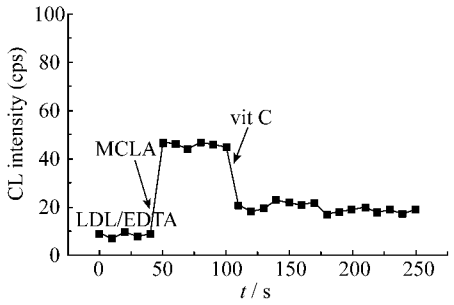

Fig. 2 MCLA-mediated chemiluminescence in LDL-EDTA-vit C system in 0.01 mol/L PBS of pH 7.2
LDL (200 mg/L) pretreated with EDTA (0.2 mmol/L). MCLA (2 μmol/L) and vit C (1 mmol/L) were added at the point indicated by the arrow. CL, chemiluminescence.

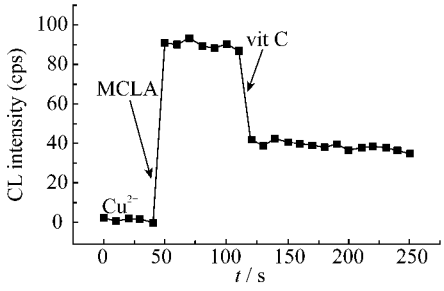

Fig. 3 MCLA-mediated chemiluminescence in Cu^{2+}-vit C system in 0.01 mol/L PBS of pH 7.2
Cu^{2+} (5 μmol/L). MCLA (2 μmol/L) and vit C (1 mmol/L) were added at the point indicated by the arrow. CL, chemiluminescence.

with EDTA instead of preincubated with Cu^{2+} (Fig. 2) or LDL was omitted from reaction solution (Fig. 3) prior to the addition of vit C.

2.2 Effect of quenchers on vit C-indueced chemiluminescence

The addition of the $O_2^{\cdot-}$ scavenger SOD and the $\cdot OH$ scavenger mannitol to the above system, prior to the reaction, didn't cause a very significant decrease (decreased by 22.5% and 8.2% respectively compared with control) in peak chemiluminescence intensity. Results were shown in Fig. 4. Considering the excellent selectivity of MCLA to $O_2^{\cdot-}$ and $^1O_2$[4], the results suggested that the reaction of vit C plus LDL exposed to Cu^{2+} mostly elicit the formation of $^1O_2$ but not $O_2^{\cdot-}$ or $\cdot OH$.

NaN_3 can be used as a quencher of $^1O_2$. In order to confirm the $^1O_2$ formation further, the effect of NaN_3 on the MCLA-mediated chemiluminescence was observed. The peak chemiluminescence intensity was inhibited markedly by 72.8% (Fig. 4).

2.3 Correlation of chemiluminescence intensity and CD concentration

The preceding observations of MCLA-mediated chemiluminescence suggested that lipid peroxidation of LDL by Cu^{2+} was a prerequisite for vit C-induced

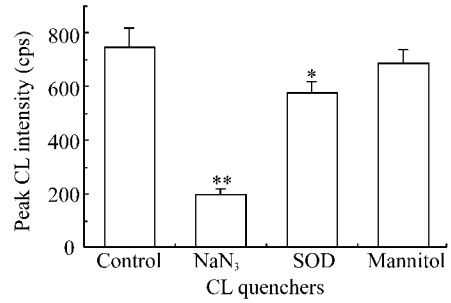

Fig. 4 Effects of the $O_2^{\cdot-}$ scavenger SOD (1 μmol/L), the $\cdot OH$ scavenger mannitol (10 mmol/L) and the $^1O_2$ quencher NaN_3 (1 mmol/L) on the vit C-induced chemiluminescence intensity in LDL-Cu^{2+}-MCLA system
LDL (200 mg/L) preoxidized for 2 h with Cu^{2+} (5 μmol/L) at 37 °C. The quenchers were added before the addition of vit C (1 mmol/L). Data are presented as $\bar{x} \pm s$ of three separate experiments. $^*P < 0.05$ in comparison with control; $^{**}P < 0.01$ in comparison with control. CL, chemiluminescence.

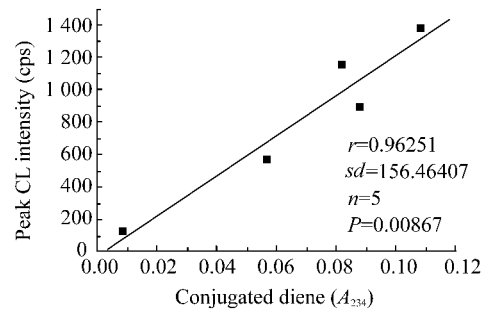

Fig. 5 The relationship of vit C-induced chemiluminescence intensity in LDL-Cu^{2+}-MCLA system and the concentration of CD
Lipid peroxidation of LDL was modulated by using different incubation times (0 h, 2 h, 4 h, 6 h and 8 h) at 37 °C then chemiluminescence and CD measurements were performed at intervals of 2 h for a period of 8 h. Other details are described in the "Materials and Methods".

light emission in the presence of MCLA. To verify the suggestion further, the changes of CD concentration and chemiluminescence intensity were investigated in a set of experiments at the same time, where LDL lipid peroxidation to different extents was used. Fig. 5 shows the relationship among the CD concentration of samples, which contained LDL and Cu^{2+} and were incubated for varying time, and the chemiluminescence peak intensity obtained after the addition of vit C.

In Fig. 5, from the linear fit of five experimental points, the correlation coefficient r could be calculated ($r \approx 0.96$). Because the extent of CD formation represents the extent of LDL oxidation[12], the high correlation coefficient between chemiluminescence intensity and CD formation indicated that $^1O_2$ generation is associated with the reaction of vit C and oxidized LDL.

3 Discussion

Dimole $^1O_2$ emissions at 634 nm and 704 nm often have been used for the identification of $^1O_2$ in biological system, but unfortunately the red emission is extremely inefficient in water[4]. On the other hand, most of the $^1O_2$ generated could be trapped by MCLA at the rate constant of $2.9 \times 10^9 (mol/L)^{-1} \cdot s^{-1}$ to produce an excited dioxetane analog, which then emits light at 465 nm with high efficiency[15]. The present study clearly indicated that MCLA-mediated chemiluminescence is a very useful and sensitive method to detect $^1O_2$ generated in biological systems.

The use of Cu^{2+} as a LDL oxidation catalyst has been justified[16] because it is possibly involved in LDL oxidation *in vivo*, as suggested by the observation that transition metal ions (mainly copper and iron) were found in atherosclerotic lesion in free and protein-bound form[17]. Cu^{2+} is the most typical oxidant to study LDL oxidation *in vitro*[9,6]. Transition metal ions can stimulate lipid peroxidation and also the decomposition of lipid hydroperoxides, thereby increasing peroxyl radical concentration. As a reductant, vit C has long been known to undergo one-electron transfer to transition metal ions, and become oxidized to the ascorbyl radical.

One pathway of termination of lipid peroxidation is the bimolecular interaction of peroxyl radicals (Russell mechanism), which gives rise to the formation of $^1O_2$ and excited carbonyls compounds, respectively. The generation of $^1O_2$ by reaction between two peroxyl radicals has previously been confirmed by its dimole emission spectrum[12]. The Russell-type termination of lipid peroxidation is elicited as long as lipid hydroperoxides are present and a reductant, such as vit C, keeps transition metals in the reduced form.

The experimental results and the relevant analysis suggest that the plausible reaction scheme in the system studied is as following:

The increase of MCLA-mediated light emission after the addition of vit C seems to result from increased formation of $^1O_2$ precursor LOO·.

$$LOO\cdot + LOO\cdot \rightarrow LOH + LC{=}O^* + {}^1O_2 \quad (1)$$

LOO· may be formed from an interaction of LOOH with LO· and vit C radical, respectively.

$$LOOH + \text{vit C radical} \rightarrow LOO\cdot + \text{vit C} \quad (2)$$

$$LOOH + LO\cdot \rightarrow LOO\cdot + LOH \quad (3)$$

The presence of vit C radical greatly accelerates the direct formation of lipid peroxyl radicals via Equation 2. Because the highly reactive LO· radicals would easily oxidize LOOH to LOO· via Equation 3, a pathway of increasing LO· formation by Cu^+ coupled with vit C-mediated Cu^{2+} reduction is most likely involved (Equation 4 and Equation 5).

$$LOOH + Cu^+ \rightarrow LO\cdot + OH^- + Cu^{2+} \quad (4)$$

$$\text{Vit C} + Cu^{2+} \rightarrow \text{vit C radical} + Cu^+ + H^+ \quad (5)$$

The process of Equation 4 is analogous to the classical Fenton reaction. Such a reaction, depending on the copper and its concentration, most likely acted as the initiator of LDL lipid peroxidation in our system. Vit C kept this reaction running upon recycling of oxidized copper ions.

As a chain-breaking antioxidant, vit C probably also reduces LO· to LOH.

$$LO\cdot + \text{vit C} \rightarrow LOH + \text{vit C radical} \quad (6)$$

This will interrupt the chain propagation of lipid peroxidation. In addition, scavenging of alkoxy radicals with vit C (Equation 6) would stimulate LOO· formation by Equation 2, and reduce LOO· formation by Equation 3. The phenomenon of MCLA-mediated chemiluminescence decreasing rapidly after reaching the maximum probably is related with Equation 6.

In summary, depending on the MCLA-mediated chemiluminescence intensity and its relation with the extent of lipid peroxidation in LDL (represented by the CD measurements), bimolecular reaction of peroxyl radicals (Russell mechanism, see Equation 1) is the most likely reaction scheme responsible for vit C-induced $^1O_2$ formation in LDL-Cu^{2+} system.

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MCLA 为化学发光探针测定氧化低密度脂蛋白中维生素 C 诱导的单线态氧产生

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摘要 以一种海萤荧光素类似物 MCLA〔2-methyl-6-(*p*-methoxyphenyl)-3, 7-dihydroimidazo[1, 2-a]pyrazin-3-one〕作为高灵敏且有选择性的化学发光探针,用化学发光的方法直接观测到了少量 Cu^{2+} 氧化的低密度脂蛋白(Ox-LDL)中维生素 C 诱导的单线态氧($^1O_2$)的产生。实验中通过叠氮化钠(NaN_3)对 MCLA 介导的化学发光的猝灭作用进一步证实了上述体系中 $^1O_2$ 的形成。根据实验观察的结果,分析了这一体系中 $^1O_2$ 形成的可能途径,认为首先是维生素 C 将 Cu^{2+} 转变为还原态,而自身失去一个电子转变为维生素 C 自由基,从而刺激了过氧自由基和烷氧自由基的形成,过氧自由基的双分子反应很可能就是体系内 $^1O_2$ 产生的反应机制。

关键词 维生素 C; 单线态氧; MCLA; 低密度脂蛋白; Cu^{2+}

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基因分析新技术及其应用学术交流会通知

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