

# 1,2-Diacylglycerol hydroperoxides induce the generation and release of superoxide anion from human polymorphonuclear leukocytes

Yorihiko Yamamoto<sup>a,\*</sup>, Yasuhiro Kambayashi<sup>b</sup>, Takashi Ito<sup>c</sup>, Keiichi Watanabe<sup>d</sup>,  
Minoru Nakano<sup>b</sup>

<sup>a</sup>Research Center for Advanced Science and Technology, University of Tokyo, 4-6-1 Komaba, Meguro-ku, Tokyo 153, Japan

<sup>b</sup>Department of Photon and Free Radical Research, Japan Immunoresearch Laboratories, 351-1 Nishiyokote Cho, Takasaki 370, Japan

<sup>c</sup>Department of Neurology, Tokyo Metropolitan Matsuzawa Hospital, Kamikitazawa, Setagaya-ku, Tokyo 156, Japan

<sup>d</sup>Department of Pathology, Tokai University School of Medicine, Isehara 259-11, Japan

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**Abstract** We examined the effect of 1-palmitoyl-2-linoleoylglycerol (PLG), PLG hydroxide (PLG-OH), and PLG hydroperoxide (PLG-OOH) on the release of superoxide anion from human PMNs monitored by the chemiluminescence generated by the superoxide anion-sensitive reagent, 2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo[1,2-*a*]pyrazin-3-one (MCLA). PLG-OOH at low micromolar concentrations stimulated human PMNs whereas PLG and PLG-OH did not. 1,3-Dilinoleoylglycerol hydroperoxide, 1-palmitoyl-2-linoleoylphosphatidylcholine hydroperoxide, and linoleic acid hydroperoxide were much less efficient in stimulating human PMNs than PLG-OOH. The PKC inhibitors, chelerythrine chloride and staurosporine, inhibited the stimulation of PMNs. Possible pathophysiological role of 1,2-diacylglycerol hydroperoxides is discussed.

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**Key words:** Diacylglycerol hydroperoxide; Human polymorphonuclear leukocyte; Superoxide anion; 2-Methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo[1,2-*a*]pyrazin-3-one; Protein kinase C

## 1. Introduction

Protein kinase C (PKC) plays a crucial role in receptor-mediated signal transduction affecting a diverse range of cellular responses such as cell proliferation, differentiation, and tumor promotion [1,2]. PKC is known to be activated by calcium ion and lipids such as phosphatidylserine and 1,2-diacylglycerol [1,2], and directly by phorbol 12-myristate 13-acetate (PMA) [3]. We have recently reported that 1,2-dilinoleoylglycerol hydroperoxide (1,2-LLG-OOH) and hydroxide (1,2-LLG-OH) activate PKC isolated from rat brain almost as efficiently as does PMA [4]. 1,2-LLG-OOH and 1,2-LLG-

OH can activate PKC even in the absence of calcium ion and phosphatidylserine [4]. It is therefore suggested that 1,2-diacylglycerol hydroperoxide (1,2-DAG-OOH) and hydroxide (1,2-DAG-OH) may activate the PKC-dependent signal transduction system if these components are released from oxidized biomembranes by the action of phospholipase C [4].

To demonstrate that oxidized 1,2-diacylglycerol can act as a biochemical messenger in cellular systems, we have investigated its activation of human polymorphonuclear leukocytes (PMN), since they are known to be activated by PMA [5] and since oxidized 1,2-diacylglycerol acts like PMA [4]. The activation of PMNs was assessed by measuring the superoxide anion released from PMNs. The production of superoxide anion was measured by a chemiluminescence method using a sea-firefly *cypridina* luciferin analogue, 2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo[1,2-*a*]pyrazin-3-one (MCLA), as a luminescence reagent [6].

## 2. Materials and methods

### 2.1. Materials

1-Palmitoyl-2-linoleoylphosphatidylcholine (PLPC), 1,3-dilinoleoylglycerol (1,3-LLG), *Bacillus cereus* phospholipase C (type XI), soybean lipoxidase (type 1B), PMA, chelerythrine chloride, *tert*-butyl hydroperoxide, and superoxide dismutase (SOD) were purchased from Sigma Chemical Co. Staurosporine and 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7) were purchased from Funakoshi (Tokyo). Linoleic acid hydroperoxide was prepared as previously described [7]. MCLA was purchased from Tokyo Kasei Kogyo (Tokyo). Solvents and other reagents were of the highest analytical grade available.

### 2.2. Preparation of diacylglycerol and its hydroperoxide and hydroxide

PLPC hydroperoxide (PLPC-OOH) and its hydroxide (PLPC-OH) were prepared as described previously [8]. PLPC, PLPC-OOH, and PLPC-OH were individually hydrolyzed with *Bacillus cereus* phospholipase C in 50 mM aqueous Tris-HCl (pH 7.4)/methanol (60:40, v/v) containing 5 mM calcium chloride at 37°C for 60 min. The resulting 1-palmitoyl-2-linoleoylglycerol (PLG), PLG hydroperoxide (PLG-OOH), and PLG hydroxide (PLG-OH) were extracted with chloroform/methanol (2:1, v/v) and the lower layer was concentrated using a rotary evaporator. PLG, PLG-OOH, and PLG-OH were purified by HPLC (Superiorex ODS column; 20×250 mm, 5 µm, Shiseido, Japan) using methanol/2-propanol (85:15, v/v) as the mobile phase at a flow rate of 8 ml/min; their elution times were 33.7, 16.7, and 16.7 min, respectively. 1,3-LLG hydroperoxide (1,3-LLG-OOH) was prepared by the aerobic autooxidation of 1,3-LLG for 3 days, and was purified from unreacted 1,3-LLG by HPLC as described above.

The concentration of hydroperoxides was determined by using a hydroperoxide-specific, isoluminol chemiluminescence assay [7]. The absence of hydroperoxide in PLG-OH, PLG, and 1,3-LLG was also confirmed by this method. The concentration of PLG-OH was estimated by its absorbance at 234 nm using an assumed molar extinction

\*Corresponding author. Fax: (81) 3-3397-6769.

E-mail: junkan@oxygen.rcast.u-tokyo.ac.jp

**Abbreviations:** PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; 1,2-LLG-OOH, 1,2-dilinoleoylglycerol hydroperoxide; 1,2-LLG-OOH, 1,2-dilinoleoylglycerol hydroxide; 1,2-DAG-OOH, 1,2-diacylglycerol hydroperoxide; 1,2-DAG-OH, 1,2-diacylglycerol hydroxide; PMNs, polymorphonuclear leukocyte(s); MCLA, 2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo[1,2-*a*]pyrazin-3-one; PLPC, 1-palmitoyl-2-linoleoylphosphatidylcholine; PLPC-OOH, PLPC hydroperoxide; PLPC-OH, PLPC hydroxide; 1,3-LLG, 1,3-dilinoleoylglycerol; 1,3-LLG-OOH, 1,3-LLG hydroperoxide; SOD, superoxide dismutase; PLG, 1-palmitoyl-2-linoleoylglycerol; PLG-OOH, PLG hydroperoxide; PLG-OH, PLG hydroxide; HBSS, Hanks' balanced salt solution; HPLC, high performance liquid chromatography; MLI, maximal luminescence intensity; cpm, counts/min

coefficient,  $28\,000\text{ M}^{-1}\text{ cm}^{-1}$  [9]. The concentration of PLG and 1,3-LLG was calculated from their weights.

### 2.3. Preparation of granulocytes

Peripheral blood from a healthy human volunteer was drawn into a syringe containing heparin (20 U/ml). Leukocytes were isolated by sedimentation in the presence of 2.0% dextran followed by brief hypotonic lysis of residual erythrocytes [10]. The resultant leukocytes ( $1.0 \times 10^7$  cells/ml) containing >90% PMN were suspended in Hanks' balanced salt solution (HBSS) containing 1.3 mM calcium ion and 0.9 mM magnesium ion, and were kept at  $0^\circ\text{C}$  for no longer than 3 h prior to use. Cell viability was determined by the trypan blue exclusion method [10].

### 2.4. Incubation system and the activation of PMNs

A glass cuvette containing PMNs ( $1.5$  or  $2.5 \times 10^4$  cells/ml) and MCLA ( $1\text{ }\mu\text{M}$ ) in 2.0 ml of HBSS was placed in a luminescence reader (Type BLR-301, Aloka, Tokyo) and the emission of light was recorded [11]. After keeping the cuvette at  $37^\circ\text{C}$  under aerobic conditions for 3 min,  $10\text{ }\mu\text{l}$  of a  $1.6\text{ mM}$  PLG-OOH in methanol (final concentration:  $8\text{ }\mu\text{M}$ ) was added to the cuvette by a microsyringe. PLG-OH, PLG, 1,3-LLG-OOH, and 1,3-LLG were added similarly instead of PLG-OOH.  $20\text{ }\mu\text{l}$  of a  $3.2\text{ }\mu\text{M}$  PMA in dimethyl sulfoxide/HBSS (1:1, v/v) was also added in a control experiment. After the addition of stimulants, SOD (final concentration:  $0.5\text{ }\mu\text{M}$ ) was added to assess the role of superoxide anion in MCLA-dependent light emission from PMNs. PKC inhibitors were added 5 min before the addition of PLG-OOH or PMA.

## 3. Results and discussion

Fig. 1A shows that the addition of PLG-OOH ( $8\text{ }\mu\text{M}$ ) immediately induced a strong light emission from PMNs in the presence of MCLA at  $37^\circ\text{C}$  under aerobic conditions. This chemiluminescence is dependent on MCLA, since no detectable photon emission was observed in the absence of MCLA (data not shown). The chemiluminescence intensity sharply increases to reaching a maximum and then the signal decays with time. The stimulant-induced maximal luminescence in-

tensity (MLI) was calculated by subtracting the baseline luminescence intensity from the maximal intensity as shown in Fig. 1A. The addition of a catalytic amount of SOD ( $0.5\text{ }\mu\text{M}$ ) suppressed the chemiluminescence intensity to a level which was smaller than the baseline luminescence intensity, indicating the involvement of superoxide anion in the MCLA-dependent chemiluminescence from human PMNs.

PMA also stimulated the MCLA-dependent light emission from PMNs and this was suppressed by the addition of SOD (Fig. 1B), as observed previously [6]. Superoxide anion can reduce the oxidized form of cytochrome *c* [12] and the initial rate (maximal rate) of the reduction can be determined by measuring the increase in absorption of the reduced form of cytochrome *c* at  $550\text{ nm}$  ( $\epsilon_{\text{max}} = 2.11 \times 10^4\text{ M}^{-1}\text{ cm}^{-1}$ ) [12]. It was found that PMA-stimulated MLI at  $10^5$  counts/min (cpm) corresponded to the release of  $0.095\text{ nmol}$  superoxide anion per minute. Thus, it was calculated that  $8\text{ }\mu\text{M}$  PLG-OOH induced the release of superoxide anion from PMNs ( $3 \times 10^4$  cells) at a maximal rate of  $0.36\text{ nmol/min}$ . It is noteworthy that cytochrome *c* cannot be used for the measurement of superoxide anion production from PMNs stimulated by PLG-OOH since cytochrome *c* rapidly decomposes PLG-OOH (unpublished data).

Next, we examined the effect of PLG-OOH concentration on MCLA-dependent light emission from PMNs. As shown in Fig. 2, MLI from PMNs in the presence of MCLA increased with increasing PLG-OOH concentration to a maximum level at about  $4\text{ }\mu\text{M}$ . Fig. 3 shows that  $8\text{ }\mu\text{M}$  PLG-OOH induced the MCLA-dependent light emission from PMNs more strongly than the same concentration of 1,3-LLG-OOH. Moreover, PLPC-OOH, linoleic acid hydroperoxide, tert-butyl hydroperoxide, and hydrogen peroxide were much less efficient in stimulating human PMNs than PLG-OOH (data not shown), suggesting that the chemical structure of 1,2-di-

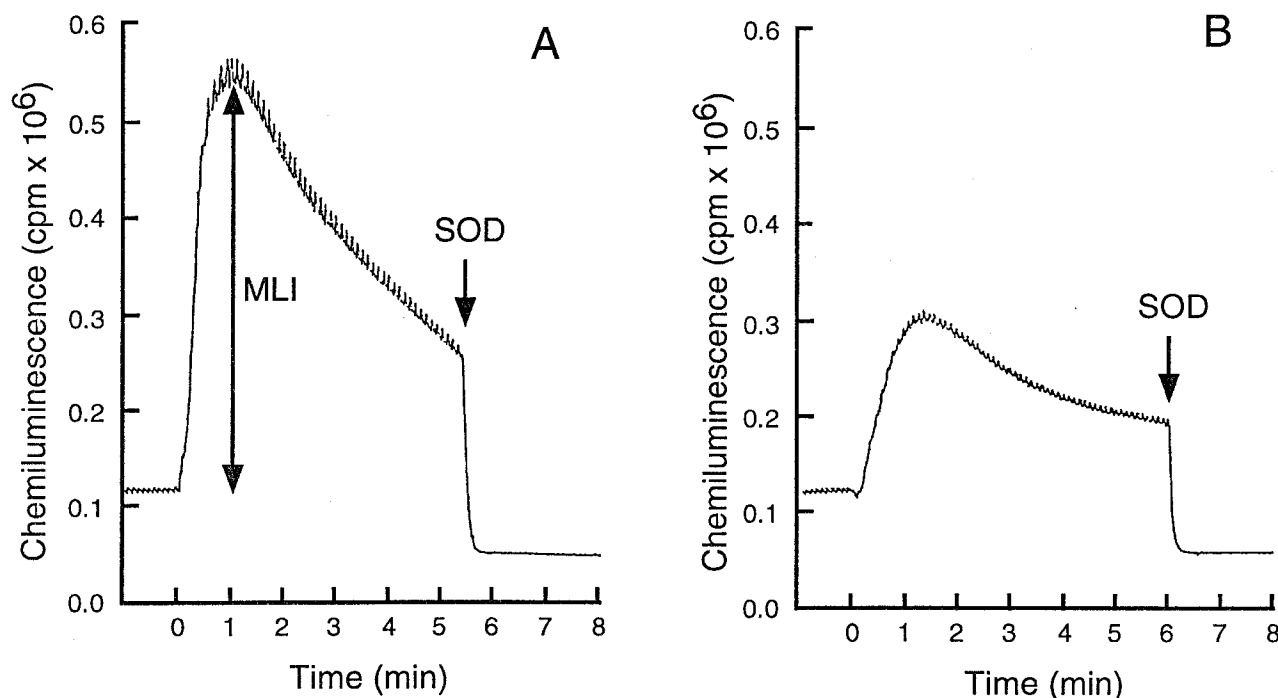


Fig. 1. Light emission from human PMNs ( $1.5 \times 10^4$  cells/ml) stimulated by (A)  $8\text{ }\mu\text{M}$  PLG-OOH and (B)  $32\text{ nM}$  PMA in the presence of  $1\text{ }\mu\text{M}$  MCLA in HBSS at  $37^\circ\text{C}$  under aerobic conditions. The stimulant was added at  $t=0$  and the addition of SOD ( $0.5\text{ }\mu\text{M}$ ) is indicated by the arrows.

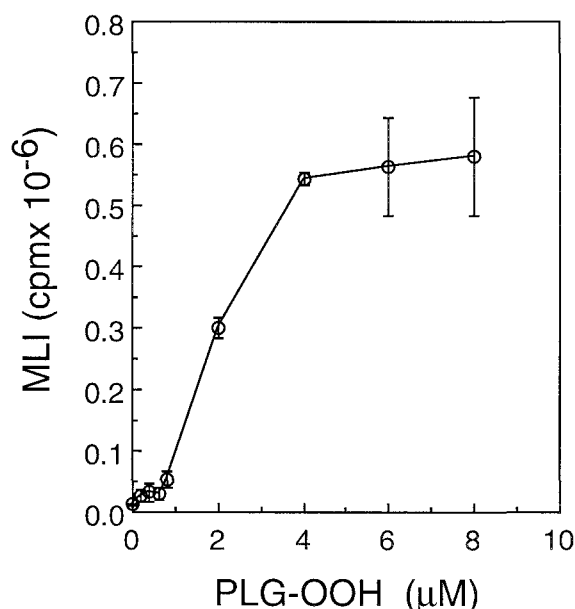


Fig. 2. The effect of PLG-OOH concentration on MLI from human PMNs ( $2.5 \times 10^4$  cells/ml) in the presence of 1  $\mu$ M MCLA in HBSS at 37°C under aerobic conditions. Vertical bars indicate standard deviation ( $n = 3$ ).

acylglycerol hydroperoxide is an essential feature for the stimulation of PMNs. Fig. 3 also shows that 1,3-LLG, PLG, and PLG-OH did not stimulate the release of superoxide anion from PMNs as efficiently as PLG-OOH. There was a significant difference between PLG-OOH and PLG-OH with respect to stimulation of PMNs. We also observed that PLG-OOH activated rat brain PKC more efficiently than PLG-OH did (S. Takekoshi et al., unpublished data) although 1,2-LLG-OOH and 1,2-LLG-OH activated the rat brain PKC with almost the same potency [4].

In order to verify the involvement of PKC in the stimulation of PMNs by PLG-OOH, the effects of PKC inhibitors, chelerythrine chloride (20  $\mu$ M) [13], staurosporine (0.5  $\mu$ M) [14,15], and 1-(5-isoquinolylsulfonyl)-2-methylpiperazine dihydrochloride (H-7, 200  $\mu$ M) [15], were examined. These inhibitors did not affect the viability of PMNs and were added to PMNs 5 min before the addition of stimulants, PLG-OOH

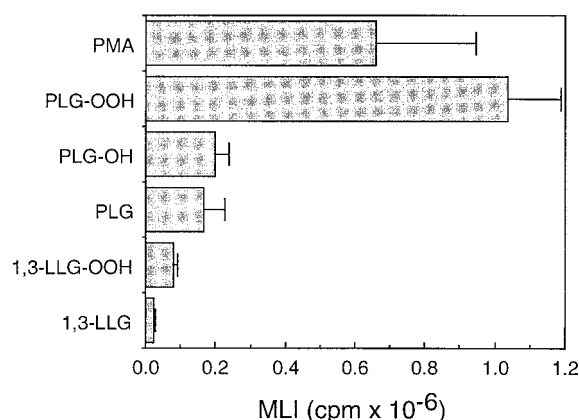


Fig. 3. MLI from human PMNs ( $2.5 \times 10^4$  cells/ml) stimulated by 8  $\mu$ M lipid derivatives or 32 nM PMA in the presence of 1  $\mu$ M MCLA in HBSS at 37°C under aerobic conditions. Horizontal bars indicate standard deviation ( $n = 3$ ).

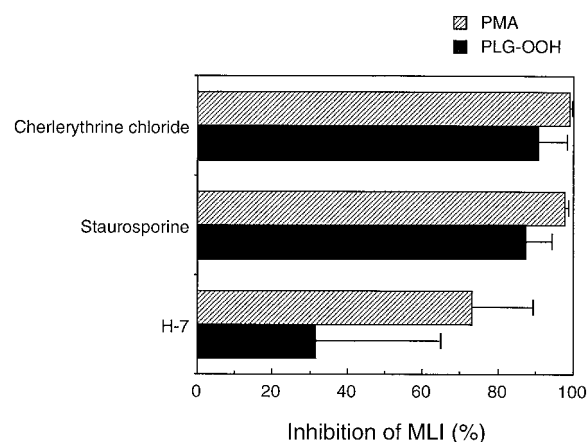


Fig. 4. Effects of PKC inhibitors (20  $\mu$ M chelerythrine chloride, 0.5  $\mu$ M staurosporine, and 200  $\mu$ M 1-(5-isoquinolylsulfonyl)-2-methylpiperazine dihydrochloride (H-7) on 32 nM PMA or 8  $\mu$ M PLG-OOH induced light emission from human PMNs ( $2.5 \times 10^4$  cells/ml) in the presence of 1  $\mu$ M MCLA in HBSS at 37°C under aerobic conditions. Horizontal bars indicate standard deviation ( $n = 3$ ).

and PMA. As shown in Fig. 4, chelerythrine chloride and staurosporine nearly suppressed all PMA-induced MLI from human PMNs while H-7 was effective by about 70%. These PKC inhibitors also suppressed PLG-OOH-stimulated MLI from human PMNs but to a lesser extent than stimulated by PMA (Fig. 4). These observation suggest that PLG-OOH stimulates PMNs primarily by activating PKC, but also that other activation system may be involved. It is also important to identify which PKC isozyme is responsible for PLG-OOH-induced PMN activation; further investigation is being considered.

Phosphatidylcholine-specific phospholipase C (PC-PLC), which catalyzes the hydrolysis of phosphatidylcholine to 1,2-diacylglycerols, has been found in PMNs, lymphocytes, and hepatic cells [16]. *Bacillus cereus* PC-PLC can hydrolyze oxidized and unoxidized phosphatidylcholine [17]. This was also confirmed in this study since PLG, PLG-OH, and PLG-OOH were prepared by the hydrolysis of PLPC, PLPC-OH, and PLPC-OOH, respectively, using *Bacillus cereus* PC-PLC. Although it is necessary to demonstrate that the mammalian PC-PLC hydrolyzes the oxidized phosphatidylcholine, 1,2-DAG-OOH are expected to be formed as a result of oxidative stress. In fact, the oxidation of hepatocyte phospholipids and plasma lipoproteins gave PC-OOH as the major product [18,19].

This and data from previous studies [4] provide evidence that 1,2-DAG-OOH can act like PMA as a powerful tumor promoter. If the production of phospholipid hydroperoxides exceeds the capacity of hydroperoxide-reducing activity at or near an inflammation site, and phospholipase C is released from phagocytic cells or other sources, then 1,2-DAG-OOH may prevail and function as a physiological tumor promoter. It should be noted that chronic inflammation is attributed as one of the major cause of cancer [20].

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