

Various Molecular Species of Diacylglycerol Hydroperoxide Activate Human Neutrophils via PKC Activation

Yasuhiro Kambayashi^{1,2,*}, Susumu Takekoshi³, Yutaka Tanino⁴, Keiichi Watanabe^{3,†}, Minoru Nakano^{2,§}, Yoshiaki Hitomi¹, Tomoko Takigawa⁵, Keiki Ogino^{1,5}, and Yorihiro Yamamoto⁴

¹Department of Environmental and Preventive Medicine, Graduate School of Medical Science, Kanazawa University, 13-1 Takara-machi, Kanazawa 920-8640, Japan

²Department of Photon and Free Radical Research[¶], Japan Immunoresearch Laboratories, 351-1 Nishiyokote-cho, Takasaki 370-0021, Japan

³Department of Pathology, Tokai University School of Medicine, Bosedai, Isehara, Kanagawa 259-1193, Japan

⁴School of Bionics, Tokyo University of Technology, 1404-1 Katakura, Hachioji 192-0982, Japan

⁵Department of Public Health, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Okayama 700-8558, Japan

Received 7 December, 2006; Accepted 22 December, 2006

Summary We have proposed that diacylglycerol hydroperoxide-induced unregulated signal transduction causes oxidative stress-related diseases. In this study, we investigated which molecular species of diacylglycerol hydroperoxide activated human peripheral neutrophils. All diacylglycerol hydroperoxides, diacylglycerol hydroxides, and diacylglycerols tested in the present study induced superoxide production by neutrophils. The ability to activate neutrophils among molecular species containing the same fatty acid composition was as follows; diacylglycerol hydroperoxide > diacylglycerol hydroxide ≥ diacylglycerol. The diacylglycerol hydroperoxide composed of linoleate was a stronger activator for neutrophils than that composed of arachidonate. 1-Palmitoyl-2-linoleoylglycerol hydroperoxide (PLG-OOH) was the strongest stimulator for neutrophils. We reconfirmed that PLG-OOH activated protein kinase C (PKC) in neutrophils. PLG-OOH induced the phosphorylation of p47^{phox}, a substrate of PKC and a cytosolic component of NADPH oxidase, in neutrophils, as did *N*-formyl-methionyl-leucyl-phenylalanine or 4β-phorbol-12β-myristate-13α-acetate. Moreover, the time course of p47^{phox} phosphorylation was comparable to that of superoxide production. These results suggest that PLG-OOH activated intracellular protein kinase C. PLG-OOH, produced via an uncontrolled process, can act as a biological second messenger to cause inflammatory disease from oxidative stress.

Key Words: 1-palmitoyl-2-linoleoylglycerol hydroperoxide, neutrophil, oxidative stress, protein kinase C, p47^{phox}

*To whom correspondence should be addressed.

Tel: +81-76-265-2218 Fax: +81-76-234-4233

E-mail: kambayashi@pub.m.kanazawa-u.ac.jp

†Dr. K. Watanabe passed away in 2002.

§Dr. M. Nakano passed away in 2003.

¶This department no longer exists.

Introduction

Lipid oxidation products are formed by free radicals *in vivo* [1–4]. Recently, various lipid oxidation products (such as phosphatidylserine hydroperoxide, cardiolipin hydroperoxide, 4-hydroxy-2-nonenal and 8-isoprostane) have attracted much attention as signaling molecules [5–11]. For example,

phosphatidylserine hydroperoxide [5, 11] and cardiolipin hydroperoxide [5, 6] participate in the apoptotic process. We have previously reported that diacylglycerol (DAG) hydroperoxide (DAG-OOH), mainly dilinoleoylglycerol hydroperoxide, activated rat brain protein kinase C (PKC) [12] as strongly as 4 β -phorbol-12 β -myristate-13 α -acetate (PMA), a powerful PKC activator [13]. DAG-OOH would be formed via the hydrolysis of phospholipid hydroperoxide by the action of phospholipase C (PLC) [14]. Oxidized phospholipids are formed by the oxidation of membrane phospholipids, since lipids are vulnerable to free radical-induced oxidation [15]. This process is unregulated since it does not mediate a receptor in the biomembrane. PMA induces various diseases, such as cancer, via PKC activation [16, 17], but it is not a physiological molecule. Therefore, DAG-OOH is expected to work as a physiological PKC activator, which induces oxidative stress-related diseases (such as cancer, inflammatory disease, autoimmune disease, and atherosclerosis) [15].

We have also reported that 1-palmitoyl-2-linoleoylglycerol hydroperoxide (PLG-OOH) induced superoxide (O₂⁻) production by human peripheral neutrophils [18]. Since other molecular species of DAG-OOH can be formed in the biological systems, it is important to confirm which molecular species of DAG-OOH activates neutrophils. O₂⁻ is produced by human neutrophils via both PKC-dependent and -independent pathways [19, 20]. Therefore, we examined which pathway occurred in DAG-OOH-stimulated PMNs. PKC inhibitors, such as chelerythrine, staurosporine, and H-7, suppressed the production of O₂⁻ from neutrophils activated by DAG-OOH [18], but this is indirect evidence. Since p47^{phox}, a cytosolic component of NADPH oxidase, is phosphorylated by PKC when O₂⁻ is generated from neutrophils, the detection of phosphorylated p47^{phox} is direct evidence for PKC activation in neutrophils [21]. If phosphorylation of p47^{phox} is observed in DAG-OOH-stimulated neutrophils, it indicates the activation of PKC in neutrophils by DAG-OOH.

In the present study, we investigated which molecular species of DAG-OOH activate human peripheral neutrophils and the phosphorylation of p47^{phox} in neutrophils stimulated by DAG-OOH.

Materials and Methods

Materials

1-Palmitoyl-2-linoleoyl-phosphatidylcholine, 1-stearoyl-2-linoleoyl-phosphatidylcholine, 1-palmitoyl-2-arachidonoyl-phosphatidylcholine, 1-stearoyl-2-arachidonoyl-phosphatidylcholine, 1,2-dioleoylglycerol (OOG), and 1-oleoyl-2-acetyl-glycerol (OAG) were purchased from Funakoshi Co. Ltd. (Tokyo, Japan). PMA, *N*-formyl-methionyl-leucyl-phenyl-alanine (fMLP), *Bacillus cereus* PLC (type IX), and soybean lipoxygenase (type 1B) were obtained from Sigma Chemical

Co. (St. Louis, MO). 2-Methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo [1,2-*a*] pyrazin-3-one (MCLA) was obtained from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). Chelex[®]100 was purchased from Bio-Rad Laboratories, Inc. (Hercules, CA). [³²P]-Orthophosphoric acid was purchased from NEN[™] Life Science Products, Inc. (Boston, MA). Solvents and other reagents were of the highest grade available. Rabbit polyclonal anti-p47^{phox} antibody was from Dr. Naoki Okamura (Hiroshima University School of Medicine) [22].

Preparation of various molecular species of diacylglycerol, their hydroperoxides and their hydroxides

Various molecular species of DAG, DAG-OOH and DAG hydroxide (DAG-OH) were prepared as previously reported [18] with some modifications. Phosphatidylcholine (PC) was purified using reversed-phase HPLC prior to use. PC (6 μ g) was oxidized with lipoxygenase (2.6 mg) in 0.2 M borate buffer treated with Chelex 100 (pH 9, 20 ml) containing 3 mM sodium deoxycholate for 20 min by mixing vigorously at room temperature under air. If necessary, sodium borohydride was added to the reaction mixture to reduce the phosphatidylcholine hydroperoxide (PC-OOH) after the reaction was over. Solid phase extraction was performed with a Sep-Pak C18 cartridge (10 g, Waters Co., Milford, MA), which was pretreated with 100 ml of 100 μ M EDTA aqueous solution. The reaction mixture containing PC-OOH or PC hydroxide (PC-OH) was applied to the cartridge after it was equilibrated with 100 ml of water. PC-OOH or PC-OH was eluted with 100 ml of methanol after washing with 200 ml of water. PC-OOH or PC-OH was purified with reversed-phase HPLC after the eluate was concentrated with a rotary evaporator, and it was filtered with a 0.22 μ m filter (MILLEX[®]-GP; Millipore Co., Bedford, MA). The PC, PC-OOH, or PC-OH was hydrolyzed by PLC in 50 mM Tris-HCl (pH 7.4) containing 40% methanol at 37°C. The reaction was monitored with HPLC. The DAG, DAG-OOH or DAG-OH formed was extracted with chloroform/methanol (2/1, by volume) three times. After the concentration of the collected lower layer using a rotary evaporator, DAG, DAG-OOH or DAG-OH was purified by HPLC with a CAPCELLPAK C18 column (type: UG120, 20 \times 250 mm, 5 μ m, Shiseido Co. Ltd., Tokyo, Japan) and methanol/2-propanol (=17/3, by volume) as the mobile phase at a flow rate of 10 ml/min. Their elution times were as follows; 15.8 min (PLG), 20.5 min (1-stearoyl-2-linoleoylglycerol; SLG), 15.5 min (1-palmitoyl-2-arachidonoylglycerol; PAG), 19.0 min (1-stearoyl-2-arachidonoylglycerol; SAG), 14.5 min (1,3-dilinoleoylglycerol; 1,3-LLG), 9.3 min (PLG-OOH), 10.5 min (SLG-OOH), 9.3 min (PAG-OOH), 10.5 min (SAG-OOH), 8.5 min (1,3-LLG-OOH), 9.3 min (PLG hydroxide; PLG-OH), 10.3 min (SLG-OH), 9.3 min (PAG-OH) and 10.8 min (SAG-OH). DAG was monitored at 215 nm. DAG-

OH or DAG-OOH was monitored at 234 nm. The concentration of DAG-OOH was determined using a hydroperoxide-specific, isoluminol chemiluminescence assay [23]. The absence of hydroperoxide in DAG-OH and DAG was also confirmed using this assay. The concentration of DAG-OH was estimated from its absorbance at 234 nm using an assumed molar extinction coefficient, $28,000 \text{ M}^{-1}\text{cm}^{-1}$ [24]. The concentration of DAG was calculated from their weights.

Measurement of MCLA-dependent chemiluminescence

Blood was drawn from healthy volunteers. Neutrophils were prepared as described previously [18]. A glass cuvette containing neutrophils (5.0×10^4 cells) and $2 \mu\text{mol}$ of MCLA in $1,990 \mu\text{l}$ of Hanks' balanced salt solution containing 1.3 mM calcium ion and 0.9 mM magnesium ion was incubated at 37°C for 3 min under air. Thereafter, $10 \mu\text{l}$ of a stimulator, such as $800 \mu\text{M}$ or 1.6 mM DAG, DAG-OOH, or DAG-OH, or $200 \mu\text{M}$ fMLP dissolved in methanol, was added to the reaction mixture using a microsyringe. MCLA-dependent luminescence ($\text{O}_2^{\cdot-}$ production [25]) was recorded using a luminescence reader (type: BLR-301, Aloka Co. Ltd., Tokyo, Japan).

Detection of phosphorylated $p47^{\text{phox}}$

Neutrophils were suspended in calcium ion, phosphate-free buffer (10 mM HEPES, 137 mM sodium chloride, 5.4 mM potassium chloride, 5.6 mM D-glucose, 0.8 mM magnesium chloride, and 0.025% BSA, pH 7.4) at 1.0×10^7 cells/ml [26]. ^{32}P -Orthophosphate was added to the neutrophil suspension at $100 \mu\text{Ci}/10^7$ cells and the cells were incubated at 37°C for 60 min with gentle mixing every 10 min. After 3 washes, calcium ion, phosphate-free buffer was added to the pellet (1.0×10^7 cells/ml).

Neutrophils (1.0×10^7 cells/ml $\times 300 \mu\text{l}$) were suspended in phosphate-free buffer ($695 \mu\text{l}$; 10 mM HEPES, 137 mM sodium chloride, 5.4 mM potassium chloride, 1.26 mM calcium chloride, 5.6 mM D-glucose, 0.8 mM magnesium chloride, and 0.025% BSA, pH 7.4) [26]. After preincubation for 1 min at 37°C , the reaction was started by adding $5 \mu\text{l}$ of stimulator dissolved in methanol (1.6 mM PLG-OOH, $200 \mu\text{M}$ fMLP or 640 nM PMA). The reaction was stopped by centrifugation ($12,000 \text{ rpm} \times 10 \text{ s}$) at the times described in the figure legends, and the supernatant was removed. Two hundred microliters of lysis buffer ($200 \mu\text{M}$ phenylmethylsulfonyl fluoride, $3 \mu\text{M}$ leupepsin, $2 \mu\text{M}$ pepstatin, $1 \mu\text{M}$ staurosporine, 100 nM calyculin A, $100 \mu\text{M}$ *o*-vanadate, 150 mM NaCl, and 1% NP-40 in 50 mM Tris-HCl (pH 8); protease inhibitor cocktail (Boehringer Mannheim, Mannheim, Germany) plus staurosporine) was added to the pellet and mixed vigorously for 1 min. After centrifugation, $5 \mu\text{l}$ of rabbit polyclonal anti- $p47^{\text{phox}}$ antibody was added to the supernatant and the solution was rotated at 4°C for 1.5 h.

Eighty microliters of 10% protein A sepharose CL-4B in NET gel buffer (Tris buffer containing NP-40, EDTA, and gelatin) was added and the solution was rotated at 4°C for 1 h. After 3 washes of the precipitate with lysis buffer, $40 \mu\text{l}$ of SDS-PAGE sample buffer was added and the samples were boiled at 100°C for 5 min. Electrophoresis was performed with a 10% polyacrylamide gel. A low molecular weight marker (Bio-Rad Laboratories, Inc., Hercules, CA) was used. Phosphorylated protein was detected using autoradiography.

Results

Synthesis of various molecular species of DAG, DAG-OOH, and DAG-OH

We first synthesized various molecular species of DAG-OOH to investigate which molecular species of DAG-OOH activate neutrophils. PC-OOH was formed by the oxidation of PC with lipoxygenase and it was hydrolyzed with PLC to form DAG-OOH, as described in the "Materials and Methods" section (Fig. 1). 1,3-LLG-OOH was synthesized by autoxidation of 1,3-LLG with α -tocopherol. The structures of various molecular species of DAG-OOH used in the present study are shown in Fig. 2. Various molecular species of DAG-OH were synthesized via the hydrolysis of PC-OH, which was formed by the reduction of PC-OOH with sodium borohydride, with PLC (Fig. 1). Various molecular species of DAG were synthesized by the hydrolysis of PC with PLC (Fig. 1).

Activation of neutrophils by various molecular species of DAG, DAG-OOH, or DAG-OH

The activation of neutrophils by various molecular species of DAG, DAG-OOH, or DAG-OH ($4 \mu\text{M}$ each) was investigated (Fig. 3). Activation of neutrophils was evaluated using maximum MCLA-dependent luminescence intensity, which represents the maximum rate of $\text{O}_2^{\cdot-}$ formation. No light emission was observed when only methanol was added to the neutrophil suspension (data not shown). The DAG-

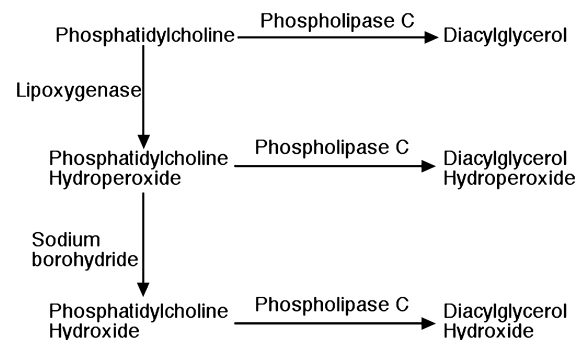


Fig. 1. Procedures for the synthesis of diacylglycerol, diacylglycerol hydroperoxide, and diacylglycerol hydroxide.

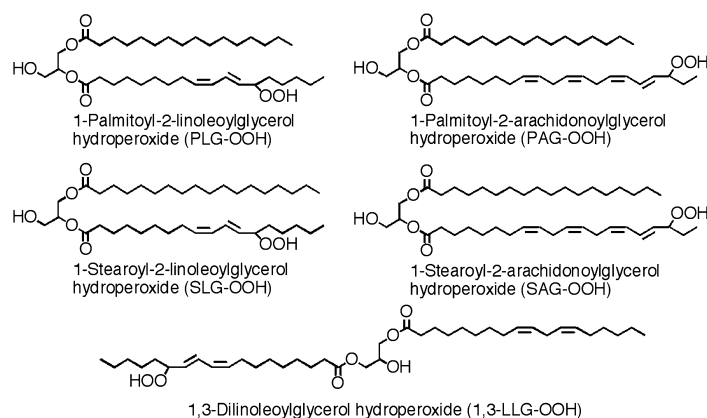


Fig. 2. Structures of various molecular species of diacylglycerol hydroperoxide used in the present study.

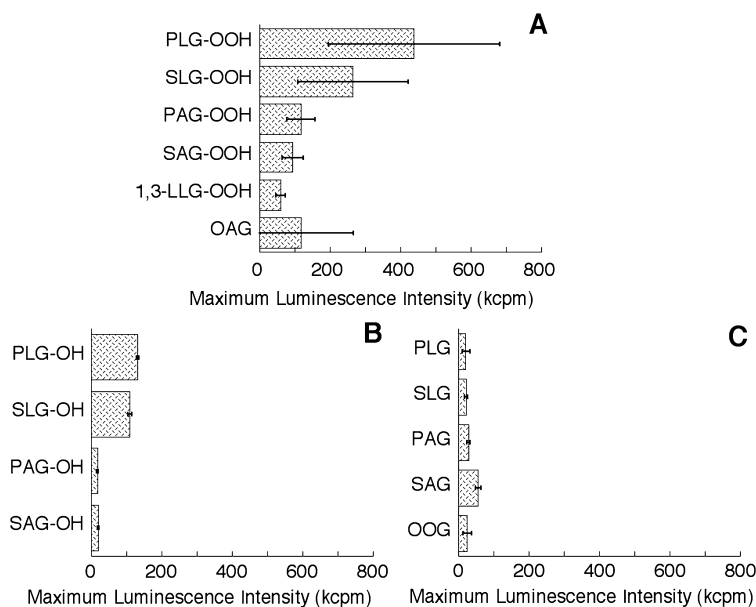


Fig. 3. Activation of neutrophils by diacylglycerol hydroperoxides (A), diacylglycerol hydroxides (B), or diacylglycerols (C). The maximum MCLA-dependent luminescence intensity was measured as the rate of superoxide anion formation from neutrophils (2.5×10^4 cells/ml). The concentration of each stimulator used here was $4 \mu\text{M}$. One micromolar MCLA was used in this experiment. Results are expressed as means \pm standard deviations ($n = 5$). Abbreviations used in the figures are as follows; PAG, 1-palmitoyl-2-arachidonoylglycerol; PLG, 1-palmitoyl-2-linoleoylglycerol; SAG, 1-stearoyl-2-arachidonoylglycerol; SLG, 1-stearoyl-2-linoleoylglycerol; 1,3-LLG, 1,3-dilinoleoylglycerol; OAG, 1-oleoyl-2-acetyl-glycerol; OOG, 1,2-dioleoylglycerol; -OOH, hydroperoxide; -OH, hydroxide.

OOH or DAG-OH composed of linoleate hydroperoxide (hydroxide) was a more powerful activator of neutrophils than that composed of arachidonate hydroperoxide (hydroxide) (Fig. 3A, B). The activation of neutrophils by 1,3-LLG-OOH was weaker than that by any 1,2-DAG-OOH, as reported previously [18]. The difference in the fatty acid composition of DAG species, including 1,2-dioleoylglycerol (OOG), which is widely used as a PKC activator, did not affect the maximum luminescence intensity (Fig. 3C). PLG-OOH was the strongest activator of neutrophils among the

various DAG, DAG-OOH, and DAG-OH species tested in the present study. Moreover, the activation of neutrophils by PLG-OOH was significantly stronger than that by 1-oleoyl-2-acetyl-glycerol (OAG), which is generally used as a stimulator of cells (Fig. 3A). Based on these findings, PLG-OOH was used in the subsequent experiments. The ability to activate neutrophils among molecular species containing the same fatty acid composition was as follows; DAG-OOH > DAG-OH \geq DAG. This order did not change when $8 \mu\text{M}$ of stimulator was used (data not shown). No significant

difference between the activation of PKC by DAG-OOH and that by DAG-OH *in vitro* has been reported [12]. Further studies are needed to elucidate why different results were obtained *in vitro* versus *ex vivo*.

Phosphorylation of p47^{phox} in neutrophils by the stimulation of DAG-OOH

The activation of PKC in neutrophils by DAG-OOH was reconfirmed. p47^{phox} is a cytosolic factor of NADPH oxidase, and known as a substrate of PKC [21]. When only methanol (0.5% to solution) was added to the neutrophil suspension, p47^{phox} was not phosphorylated after 2 min at 37°C (Fig. 4). p47^{phox} was phosphorylated in 8 μM PLG-OOH-stimulated neutrophils, as in PMA (3 nM)-stimulated or fMLP (1 μM)-stimulated neutrophils (Fig. 4).

The phosphorylated p47^{phox} at 1 min after stimulation decreased at 2 min in both fMLP (1 μM)-stimulated and PLG-OOH (8 μM)-stimulated neutrophils (Fig. 5A). This change was comparable to that in MCLA-dependent luminescence intensity (1 μM fMLP; Fig. 5B, 8 μM PLG-OOH; Fig. 5C). These results suggest that p47^{phox} was phosphorylated by PKC in PLG-OOH-stimulated neutrophils. In fact, the production of O₂⁻ was inhibited by serine/threonine kinase inhibitors and PKC inhibitors, such as staurosporine, H-7, and chelerythrine [18].

Discussion

The activation of human neutrophils by various molecular species of DAG, DAG-OOH, and DAG-OH was investigated in the present study. In order of the amount of PC in rat liver,

the species ranked as follows; SPC (16.1%)>SLPC (14.8%)>PLPC (14.1%)>PAPC (9.7%) [27]. In terms of the amount of fatty acid in the phospholipids of mammalian cells, fatty acid ranked as follows; palmitate (33%)>oleate (25%)>stearate (12%)>linoleate (10%)>arachidonate (8%) (human neutrophil phospholipids) [28], palmitate (33%)>arachidonate (19%)>stearate (16%)>oleate (13%)>linoleate (7%) (human coronary artery phospholipids) [29], and linoleate (25%)>stearate (24%)>palmitate (17%)>arachidonate (16%) (rat heart phosphatidylcholine at 100 days old) [30]. Almost all phospholipids in mammalian cells have a saturated fatty acid at the *sn*-1 position and an unsaturated fatty acid at the *sn*-2 position. Oleate is not easily oxidized by free radicals.

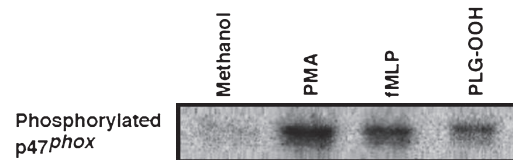


Fig. 4. Phosphorylation of p47^{phox} in neutrophils (3×10^6 cells/ml) activated by various stimulators. Lane 1; Methanol (0.5% of solution) was used in the control experiment (2 min). Lane 2; Three nanomolar 4β-phorbol-12β-myristate-13α-acetate (PMA) was used, and stimulation was stopped at 5 min. Lane 3; One micromolar *N*-formyl-methionyl-1-leucyl-phenylalanine (fMLP) was used, and stimulation was stopped at 2 min. Lane 4; Eight micromolar 1-palmitoyl-2-linoleoylglycerol hydroperoxide (PLG-OOH) was used, and stimulation was stopped at 2 min.

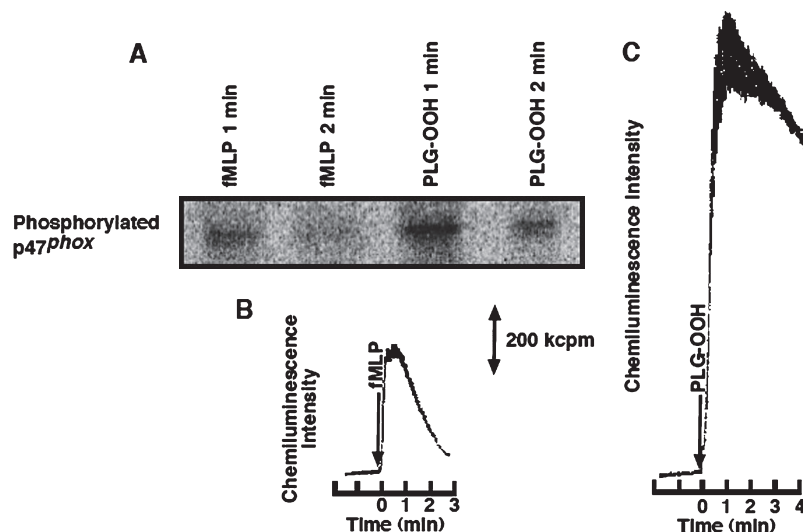


Fig. 5. Time course of p47^{phox} phosphorylation in neutrophils (3×10^6 cells/ml) stimulated by 1 μM *N*-formyl-methionyl-1-leucyl-phenylalanine (fMLP) or 8 μM 1-palmitoyl-2-linoleoylglycerol hydroperoxide (PLG-OOH) (A). Time-dependent change in MCLA-dependent luminescence from 1 μM fMLP-stimulated neutrophils (2.5×10^4 cells/ml) (B) and 8 μM PLG-OOH-stimulated neutrophils (2.5×10^4 cells/ml) (C).

Therefore, diacylglycerols with palmitate or stearate at the *sn*-1 position and linoleate or arachidonate at the *sn*-2 position were used in the present study. Although all molecular species of DAG, DAG-OOH, and DAG-OH containing these fatty acids activated human neutrophils, PLG-OOH was the most potent activator.

Human neutrophils have PKC α and PKC δ [31]. We also detected these PKC isoforms in neutrophils in the Western blot analysis (data not shown). Recently, it was found that DAG-OOH activated various PKC isoforms *in vitro*, but only PKC α and PKC δ were activated by DAG-OOH more strongly than by DAG (our unpublished data). These PKC isoforms may be involved in the activation of neutrophils by PLG-OOH.

Calcium, phospholipid-independent activity of bovine brain PKC is increased by oxidation of the regulatory domain of PKC by hydrogen peroxide or periodate [32, 33]. Even PKC in cells (PKC γ in N/N1003A cells [34] and PKC δ in COS-7 cells [35]) was oxidized by hydrogen peroxide and was activated. Oxidation of PKC by PLG-OOH might cause neutrophil activation in the present study. However, 8 μ M PC-OOH, linoleate hydroperoxide, and hydrogen peroxide did not induce production of O₂⁻ by human neutrophils in our previous study [18]. Moreover, we reconfirmed the activation of PKC in neutrophils by PLG-OOH using p47^{phox} phosphorylation in the present study. Therefore, PLG-OOH might activate PKC in neutrophils directly. PKC δ is activated via tyrosine phosphorylation by high concentrations (mM) of hydrogen peroxide [36, 37]. Further study is needed to clarify whether tyrosine residues of PKC δ in neutrophils are phosphorylated by PLG-OOH stimulation.

It has been suggested that oxidative stress causes disease, cancer, and aging through the accumulation of oxidative damage, including the oxidation of DNA, lipids, and proteins [15]. However, there is little direct evidence that oxidative damage is the cause of various diseases, although much indirect evidence has been reported. We previously proposed that DAG-OOH, formed by the actions of PLC after the oxidation of biomembrane phospholipids by free radicals [14], activates PKC, and causes diseases such as cancer, since this signal is not regulated by receptors (Fig. 6) [12, 16]. In the present study, we confirmed that PLG-OOH activates intracellular PKC. This result suggests that our proposal occurs in biological systems.

The activation of neutrophils is involved in inflammatory diseases (for example; acute inflammatory liver injury [38] and chronic inflammatory airway disease [39]). PKC is activated under oxidative stress [40] (for example; cardiac reperfusion [41], diabetic nephropathy [42] and ischemia reperfusion of skeletal muscle [43]). In some cases, PLG-OOH may activate PKC.

In summary, various molecular species of DAG, DAG-OOH and DAG-OH were synthesized. All the DAG-OOHs

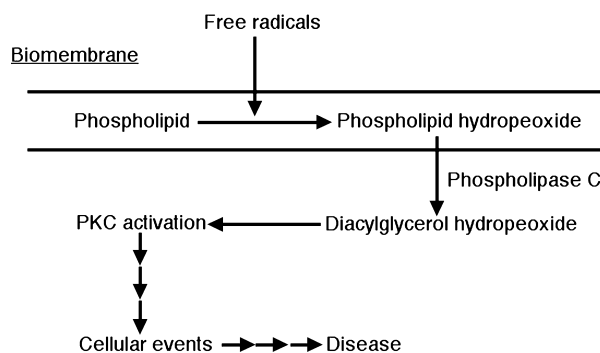


Fig. 6. Proposed mechanism for free radical-induced unregulated signal transduction.

tested in the present study activated neutrophils, but PLG-OOH was the most potent activator. When PLG-OOH stimulated neutrophils, O₂⁻ was produced via the phosphorylation of p47^{phox} by PKC. These results suggest that PLG-OOH activates PKC in cells. Therefore, PLG-OOH, produced via an uncontrolled process, can act as a second messenger in the progression from oxidative stress-induced damage to inflammatory disease (Fig. 6).

Acknowledgments

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of the Japanese Government.

Abbreviations

DAG, diacylglycerol; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; 1,3-LLG, 1,3-dilinoleoylglycerol; MCLA, 2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo [1,2-*a*] pyrazin-3-one; O₂⁻, superoxide; OAG, 1-oleoyl-2-acetyl-glycerol; OOG, 1,2-dioleoylglycerol; -OH, hydroxide; -OOH, hydroperoxide; PAG, 1-palmitoyl-2-arachidonoylglycerol; PC, phosphatidylcholine; PKC, protein kinase C; PLC, phospholipase C; PLG, 1-palmitoyl-2-linoleoylglycerol; PMA, 4 β -phorbol-12 β -myristate-13 α -acetate; SAG, 1-stearoyl-2-arachidonoylglycerol; SLG, 1-stearoyl-2-linoleoylglycerol.

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