

1-Oleoyl-2-acetyl-glycerol Stimulates 5-Lipoxygenase Activity via a Putative (Phospho)lipid Binding Site within the N-terminal C2-like Domain*

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5-Lipoxygenase (5-LO) catalysis is positively regulated by Ca²⁺ ions and phospholipids that both act via the N-terminal C2-like domain of 5-LO. Previously, we have shown that 1-oleoyl-2-acetyl-glycerol (OAG) functions as an agonist for human polymorphonuclear leukocytes (PMNL) in stimulating 5-LO product formation. Here we have demonstrated that OAG directly stimulates 5-LO catalysis *in vitro*. In the absence of Ca²⁺ (chelated using EDTA), OAG strongly and concentration-dependently stimulated crude 5-LO in 100,000 × *g* supernatants as well as purified 5-LO enzyme from PMNL. Also, the monoglyceride 1-*O*-oleyl-*rac*-glycerol and 1,2-dioctanoyl-*sn*-glycerol were effective, whereas various phospholipids did not stimulate 5-LO. However, in the presence of Ca²⁺, OAG caused no stimulation of 5-LO. Also, phospholipids or cellular membranes abolished the effects of OAG. As found previously for Ca²⁺, OAG renders 5-LO activity resistant against inhibition by glutathione peroxidase activity, and this effect of OAG is reversed by phospholipids. Intriguingly, a 5-LO mutant lacking tryptophan residues (Trp-13, -75, and -102) important for the binding of the 5-LO C2-like domain to phospholipids was not stimulated by OAG. We conclude that OAG directly stimulates 5-LO by acting at a phospholipid binding site located within the C2-like domain.

In the biosynthesis of leukotrienes (LTs)¹, 5-lipoxygenase (5-LO) catalyzes the initial oxygenation of arachidonic acid (AA) leading to 5-HPETE, which is further metabolized by 5-LO to LTA₄ (for review, see Refs. 1 and 2). The mechanisms

leading to activation of 5-LO in the cell are complex, and the enzymatic activity of 5-LO is tightly controlled. At its active site, 5-LO contains an essential nonheme-bound iron (3), which, in the resting state, is in the ferrous (Fe²⁺) form and requires oxidation to the ferric (Fe³⁺) state to enter the catalytic cycle (4, 5).

In the absence of stimulating co-factors, 5-LO activity *in vitro* is low, and it has been found that Ca²⁺, ATP, and phospholipids are required for full enzyme activity (for review, see Refs. 1 and 2). Also, a threshold level of lipid hydroperoxides (LOOH) is necessary for initial enzyme activation, converting ferrous to ferric iron (4, 6, 7). For cellular 5-LO activation, elevated Ca²⁺ levels (8), phosphorylation events by tyrosine kinases (9) and by members of the mitogen-activated protein kinase family (10, 11), an elevated peroxide tone (12–14), and nuclear membrane association (15, 16), including co-localization with the 5-LO-activating protein (17, 18), are determinants. Nevertheless, the precise pathway(s) and regulatory mechanisms of 5-LO activation in the cell are incompletely understood.

Based on theoretical models of the tertiary structure, 5-LO consists of a catalytic and an N-terminal C2-like β-barrel domain (19–22). The C2-like domain binds Ca²⁺ and phosphatidylcholine (PC) (19, 21) and targets 5-LO to the nuclear membrane (20, 21). Ca²⁺ and/or phospholipids can strongly augment 5-LO activity *in vitro* but also may have no stimulatory effect, which depends on the assay conditions (see Refs. 1 and 23 and references therein). Ca²⁺ has been shown to increase the affinity of 5-LO toward AA (24), to facilitate membrane association and binding to PC vesicles (21, 25), and appears to reduce the requirement of 5-LO for activating lipid hydroperoxides (26). For stimulation by phospholipids, apparently only the zwitterionic PC and a non-physiological cationic phospholipid (but not phosphatidylserine, phosphatidylethanolamine, phosphatidylinositol, or diacylglycerol (DAG)) stimulate 5-LO reactions (27, 28). It has been proposed that 5-LO catalysis occurs at an interface between lipid and water, and the ratio of AA to phospholipid seems important for its activity (29).

Previous studies show that DAGs similar to OAG up-regulate agonist-induced 5-LO product synthesis in leukocytes, which in part might be due to increased availability of substrate for 5-LO (see Ref. 30 and references therein). Recently, we found that OAG, not only primes leukocytes for enhanced 5-LO product synthesis upon subsequent agonist stimulation, but also functions as a direct agonist for polymorphonuclear leukocytes (PMNL), stimulating, by novel undefined mechanisms (30), 5-LO product formation in the presence of exogenously added AA. Thus, OAG-induced 5-LO activity is not connected with increased nuclear membrane association or

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¹ The abbreviations used are: LT, leukotriene; AA, arachidonic acid; DAG, 1,2-diacylglycerol; DOG, 1,2-dioctanoyl-*sn*-glycerol; EAG, 1-*O*-hexadecyl-2-acetyl-*sn*-glycerol; GPx, glutathione peroxidase; GSH, glutathione; 5-HPETE, 5(*S*)-hydro(pero)xyeicosatetraenoic acid; 5-HETE, 5(*S*)-hydroxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid; 5(*S*),12(*S*)-DiHETE, 5(*S*),12(*S*)-di-hydroxy-6,10-*trans*-8,14-*cis*-eicosatetraenoic acid; 5-LO, 5-lipoxygenase; LOOH, lipid hydroperoxide; OG, 1-*O*-oleyl-*rac*-glycerol; OAG, 1-oleoyl-2-acetyl-*sn*-glycerol; PBS, phosphate-buffered saline; PK, protein kinase; PMNL, polymorphonuclear leukocytes; S100, 100,000 × *g* supernatant; SAG, 1-stearoyl-2-arachidonoyl-*sn*-glycerol; PC, phosphatidylcholine; HPLC, high pressure liquid chromatography; wt, wild type.

Ca²⁺ mobilization, and inhibitors of kinases relevant for 5-LO activation fail to suppress the OAG effect. Here we demonstrated that OAG can directly stimulate 5-LO *in vitro*. The 5-LO stimulatory effect of OAG resembled that of Ca²⁺ and appeared to be mediated via a phospholipid binding site located on the C2-like domain of 5-LO.

EXPERIMENTAL PROCEDURES

Materials—1-O-Oleoyl-*rac*-glycerol (OG), 1-stearoyl-2-arachidonoyl-*sn*-glycerol (SAG), and 1-O-hexadecyl-2-acetyl-*sn*-glycerol (EAG) were from Alexis (Grünberg, Germany). 1,2-Dioctanoyl-*sn*-glycerol (DOG), glutathione peroxidase (GPx)-1 from bovine erythrocytes, OAG, and AA, were from Sigma. HPLC solvents were from Merck (Darmstadt, Germany).

Cells—Human PMNL were freshly isolated from leukocyte concentrates obtained at St. Markus Hospital (Frankfurt, Germany). In brief, venous blood was taken from healthy adult donors, and leukocyte concentrates were prepared by centrifugation at 4000 × *g* for 20 min at 20 °C. PMNL were immediately isolated by dextran sedimentation, centrifugation on Nycoprep cushions, and hypotonic lysis of erythrocytes, as described previously (31). Cells (10⁸/ml; purity > 96–97%) were finally resuspended in PBS containing 1 mg/ml glucose.

Site-directed Mutagenesis and Expression of 5-LO Proteins in *Escherichia coli*—Site-directed mutagenesis of the pT3-5LO plasmid, encoding wild type 5-LO (wt-5LO), using the QuikChange™ kit from Stratagene yielded the mutated 5-LO plasmid pT3-5LO-N43A-D44A-E46A (referred to as loop2 mut-5LO) (19). The codons for Trp-13, -75, and -102 were mutated in the same manner yielding the 5-LO mutant 3W mut-5LO. The mutated DNAs were confirmed using the DYEnamic ET Terminator Cycle sequencing Kit (Amersham Biosciences), followed by analysis on an Applied Biosystem PRISM 377 sequencer (carried out by KISeq, Core Facilities at Karolinska Institutet). *E. coli* MV1190 was transformed with mutated or wild type DNAs, and recombinant 5-LO proteins were expressed at 27 °C as described previously (19).

Preparation of 100,000 × *g* Supernatants and Purification of 5-LO Proteins—For purification of recombinant 5-LO proteins, *E. coli* was lysed by incubation in 50 mM triethanolamine/HCl, pH 8.0, 5 mM EDTA, soybean trypsin inhibitor (60 μg/ml), 1 mM phenylmethylsulphonyl fluoride, and lysozyme (500 μg/ml), homogenized by sonication (three times for 15 s each), and centrifuged at 19,000 × *g* for 15 min. Proteins including 5-LO were precipitated with 50% saturated ammonium sulfate during stirring on ice for 60 min. The precipitate was collected by centrifugation at 16,000 × *g* for 25 min, and the pellet was resuspended in 20 ml of PBS containing 1 mM EDTA and 1 mM phenylmethylsulphonyl fluoride. For purification of 5-LO from human PMNL, 5 × 10⁸ cells were resuspended in 10 ml of PBS containing 1 mM EDTA. After cooling on ice for 10 min, the cells were homogenized by sonication (three times for 10 s each). Whole homogenates of PMNL and the 16,000 × *g* supernatants from *E. coli* were centrifuged at 100,000 × *g* for 70 min at 4 °C, and the 100,000 × *g* supernatant (S100) was applied to an ATP-agarose column. The column was eluted as described previously (32). S100 or purified 5-LOs were immediately used for 5-LO activity assays.

Determination of 5-LO Product Formation in Cell-free Systems—To obtain whole cell homogenates of PMNL and corresponding S100, 10⁸ freshly isolated PMNL were resuspended in 1 ml of PBS containing 1 mM EDTA, cooled on ice for 10 min, and sonicated (three times for 10 s each) at 4 °C. The whole homogenate was then centrifuged (100,000 × *g* for 70 min at 4 °C) to get the S100. For determination of 5-LO activity in whole homogenates or in S100 from PMNL (7.5 × 10⁶ cells) or *E. coli*, 1 mM ATP, phospholipids, glycerides, and GPx-1/GSH were added as indicated. For determination of the activity of purified 5-LO from PMNL or *E. coli*, 5-LO (corresponding to 2 × 10⁷ PMNL or 0.5 μg of 5-LO protein, respectively) was added to 1 ml of a 5-LO reaction mix (PBS, pH 7.4, 1 mM EDTA, and 1 mM ATP). After 5 min at 4 °C, the samples containing either homogenates, S100, or partially purified 5-LO were prewarmed for 30 s at 37 °C, and AA at the indicated concentrations with or without 2 mM CaCl₂ was added to start the 5-LO reaction. For PMNL-S100 and purified 5-LO enzyme in the presence of Ca²⁺ or OAG (as well as without additives), maximum amounts of 5-LO products were formed within 6–8 min, remaining constant until ~13–15 min (not shown), implying that a 10-min incubation is a suitable time period to assess 5-LO product formation. Thus, the reaction was stopped after 10 min with 1 ml of methanol. Formed 5-LO metabolites were extracted and analyzed by HPLC, as described previously (33). 5-LO activity in samples derived from PMNL is expressed as ng of 5-LO products/10⁶ cells. In samples from *E. coli*, 5-LO activity is ex-

pressed as ng of 5-LO products/μg of protein. 5-LO products include LTB₄ (in homogenates and S100 of PMNL) and its all-*trans* isomers, 5(S),12(S)-DiHETE, 5-HETE, and 5-HPETE. 5-HETE and 5-HPETE co-elute as one major peak, and integration of this peak represents both eicosanoids. Cysteinyl LTs (LTC₄, -D₄, and -E₄) were not detected, and oxidation products of LTB₄ were not determined.

Determination of Glutathione Peroxidase Activity—GPx activity was measured according to the indirect GSH reductase-coupled method described by Wendel (34). One unit of GPx activity was defined as the conversion of 0.5 μmol of NADPH to NADP⁺/min at 37 °C and 1 mM GSH. GPx activity is expressed as milliunits/10⁶ cells.

Statistics—The program GraphPad PRISM version 3.0 was used for statistical comparisons. Statistical evaluation of the data was performed using the Student's *t* test for unequal observations. A *p* value of <0.05 was considered significant.

RESULTS

OAG Stimulates 5-LO *in Vitro* in the Absence of Ca²⁺—The effects of OAG on 5-LO activity *in vitro* were determined using whole PMNL homogenates and corresponding S100, as well as for partially purified 5-LO derived from PMNL. Under optimized assay conditions, *i.e.* the presence of 1 mM ATP and 1 mM Ca²⁺ and absence of GPx activity, OAG (10–100 μM) caused no enhancement of 5-LO product synthesis, regardless of the substrate concentration (2–40 μM AA) and the source of 5-LO (homogenates, S100, or purified 5-LO; Fig. 1A). In contrast, OAG slightly impaired 5-LO activity in a concentration-dependent manner.

To determine whether OAG could activate 5-LO under sub-optimal assay conditions, Ca²⁺ was excluded from the incubations, which resulted in reduced 5-LO activity in homogenates, in S100, and for purified 5-LO in particular at low substrate concentrations (<20 μM AA; compare also Ref. 23). For 5-LO in homogenates, the activity was not increased by OAG (0.3–100 μM) in the absence of Ca²⁺. However, crude 5-LO in S100 or purified 5-LO was concentration-dependently up-regulated by OAG up to 8- and 14-fold, respectively (Fig. 1B). Significant effects of OAG were apparent already at 0.3 μM OAG, and maximal stimulation was observed at 30 μM. Simultaneous addition of OAG together with AA caused the same stimulatory effect on 5-LO (in S100 or purified enzyme) as compared with prolonged OAG preincubation for up to 60 min prior to AA addition (not shown). Moreover, OAG was able to up-regulate the activity of 12-LO from human platelets and 15-LO from eosinophilic PMNL (~3–4-fold) in the absence of Ca²⁺, although to a lower extent as compared with 5-LO (Fig. 1C). In the presence of Ca²⁺, OAG also failed to stimulate 12- and 15-LO (not shown).

In agreement with previous observations (23), the stimulatory effect of Ca²⁺ on 5-LO activity is most pronounced when the substrate concentration is low, whereas at higher amounts of AA (≥20 μM), Ca²⁺ fails to increase 5-LO product synthesis (Fig. 1D). Similarly, OAG markedly increased 5-LO product formation at low AA concentrations (0.5–10 μM AA) but had no significant effect at AA concentrations ≥20 μM (Fig. 1E). In our previous report (26) we suggest that stimulation of 5-LO by Ca²⁺ is because of an increased affinity toward activating LOOH. In fact, the addition of 5-HPETE to PMNL-S100 concentration-dependently up-regulated the LTA₄ synthase activity of 5-LO in the absence of Ca²⁺ or OAG (Fig. 1F). However, it should be noted that 5-HPETE could simply serve as the 5-LO substrate for LTA₄ synthesis under these conditions, thereby increasing the amounts of LTA₄ hydrolysis products.

Phospholipids Abolish the 5-LO Stimulatory Effects of OAG—The fact that OAG stimulates 5-LO in S100 but not in homogenates led us to conclude that cellular membrane components of homogenates, *i.e.* phospholipids, may interfere with the action of OAG on 5-LO. S100, essentially devoid of phos-

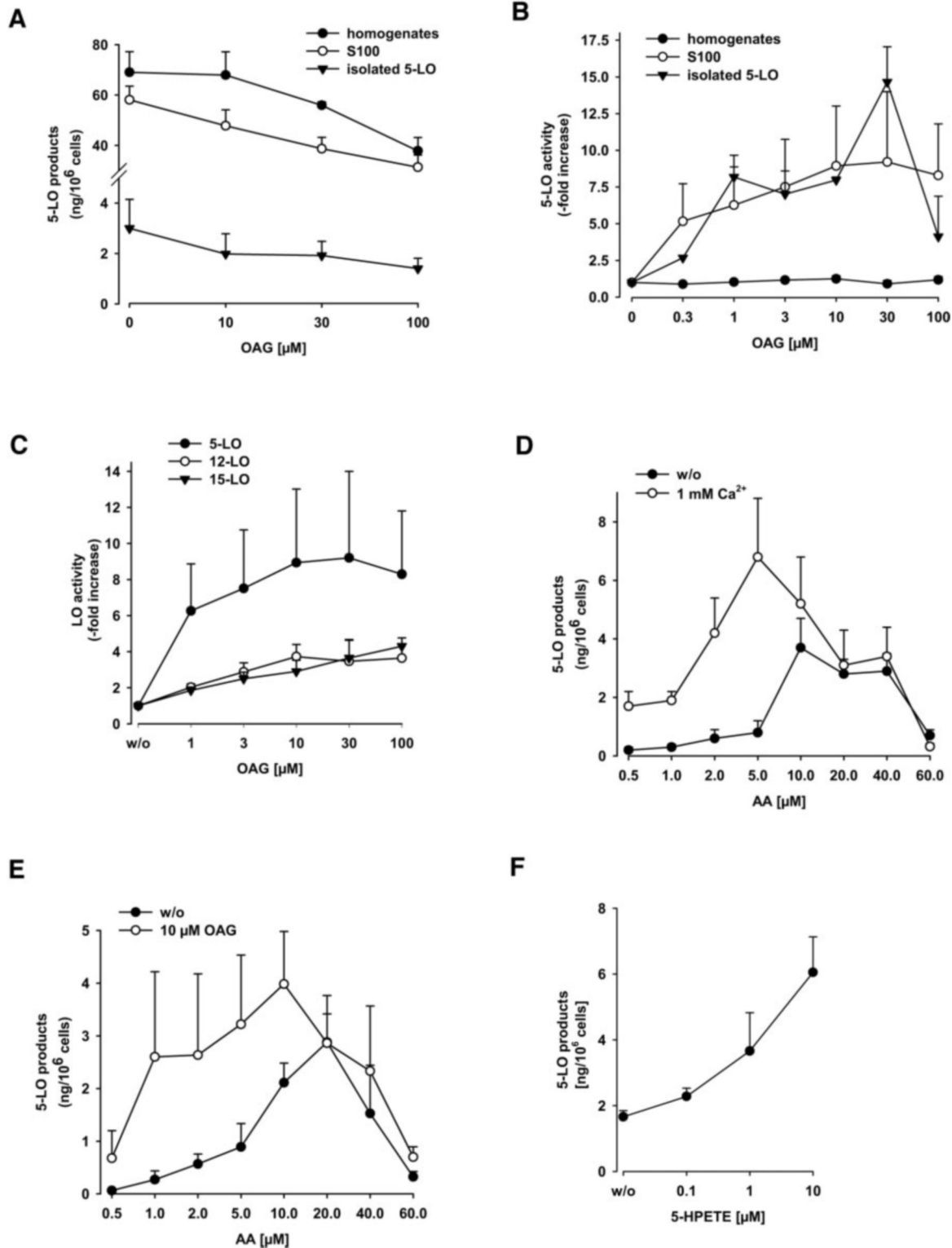


FIG. 1. OAG stimulates 5-LO activity in the absence of Ca²⁺. *A*, effects of OAG in the presence of Ca²⁺. Homogenates and S100 of isolated PMNL (7.5×10^6 cells) or 5-LO isolated from 2×10^7 PMNL were incubated in PBS plus 1 mM EDTA, 1 mM ATP, and the indicated concentrations of OAG at 4 °C. After 5 min, the samples were prewarmed, and 2 mM CaCl₂ plus 5 μ M AA was added. After 10 min at 37 °C, 5-LO activity was determined as described under "Experimental Procedures." *B*, effects of OAG in the absence of Ca²⁺. Samples were prepared and 5-LO activity was determined as described above, except that CaCl₂ was omitted. 5-LO activity in the absence of OAG (control) was 4.7 ± 1.6 , 5.9 ± 1.4 , and 0.9 ± 0.3 ng/10⁶ cells for homogenates, S100, and purified 5-LO, respectively. *C*, effects of OAG on the activity of 12- and 15-LO. 12-LO activity was determined in S100 of 10⁸ platelets. 5- and 15-LO activities were determined in S100 of isolated PMNL. The activities of the LOs in the absence of Ca²⁺ were assessed as described above. In the absence of OAG (control), the activities of the LOs were 5.9 ± 1.4 , 0.52 ± 0.06 , and 4.3 ± 0.9 ng/10⁶ cells for 5-, 12-, and 15-LO, respectively. *D* and *E*, influence of the AA concentrations on the 5-LO stimulatory effects by Ca²⁺ or OAG. 5-LO, isolated from PMNL, was diluted in PBS plus 1 mM EDTA and 1 mM ATP. The samples were either prewarmed and 2 mM CaCl₂ plus the indicated concentrations of AA were added (*D*) or preincubated with 10 μ M OAG for 5 min at 4 °C and then prewarmed, and AA was added (*E*). After 10 min, 5-LO activity was determined. *F*, effect of 5(S)-HPETE on 5-LO activity. The S100 of PMNL (7.5×10^6 cells) were incubated in PBS plus 1 mM EDTA and 1 mM ATP at 4 °C, prewarmed for 30 s at 37 °C, and the indicated concentrations of 5(S)-HPETE and 5 μ M AA were added. After 10 min at 37 °C, 5-LO activity (LTB₄ and its all-*trans* isomers) was determined. All values are given as mean \pm S.E., $n = 3-5$.

A

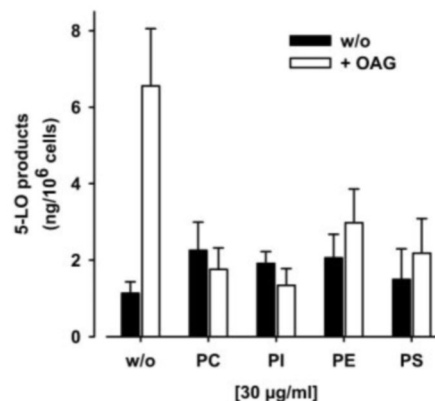
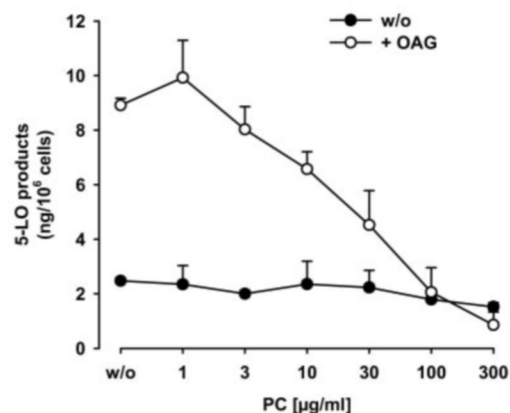
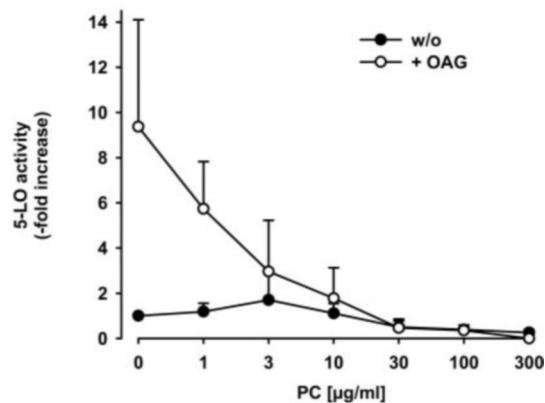


FIG. 2. **Phospholipids reverse the stimulatory effects of OAG on 5-LO activity.** A, PMNL-S100 were incubated with 1 mM ATP and the indicated phospholipids (30 μg/ml) with or without 10 μM OAG in PBS plus 1 mM EDTA at 4 °C for 5 min. The samples were prewarmed, and 5 μM AA was added. After 10 min, 5-LO activity was determined as described under "Experimental Procedures." B, PMNL-S100 were incubated with 1 mM ATP and the indicated concentrations of PC with or without 10 μM OAG in PBS plus 1 mM EDTA at 4 °C for 5 min. The samples were prewarmed, and 5 μM AA was added. After 10 min, 5-LO activity was determined. C, 5-LO, isolated from PMNL, was incubated as described for S100, and enzyme activity was determined. Values are given as mean ± S.E., *n* = 3–4.

B



C



phospholipid membranes, was supplemented with various phospholipids (phosphatidylcholine, phosphatidylinositol, phosphatidylethanolamine, and phosphatidylserine; 30 μg/ml each), and 5-LO activity was determined. All of the phospholipids suppressed the effects of OAG without inhibiting 5-LO in the absence of OAG (Fig. 2A). More detailed analysis showed that PC reduced the effects of OAG in a concentration-dependent manner ($IC_{50} \approx 20 \mu\text{g/ml}$) in S100 (Fig. 2B). For purified 5-LO (also essentially devoid of endogenous phospholipids), stimulation by OAG (10 μM) was almost completely blocked at 3 μg/ml

of exogenously added PC (Fig. 2C).

Effects of Various Glycerides on 5-LO Activity—Next, other glycerides structurally related to OAG were tested for 5-LO stimulation in PMNL-S100. In the absence of Ca^{2+} , the monoglyceride OG, the diacylglycerol DOG, as well as the ether-linked *O*-alkyl-acylglycerol EAG caused 5-LO stimulatory effects in a dose-dependent manner, although the magnitude of 5-LO activation was less pronounced as compared with OAG (Fig. 3). Interestingly, SAG, which is a long chain DAG, failed to stimulate 5-LO. As observed for OAG, no 5-LO stimulation

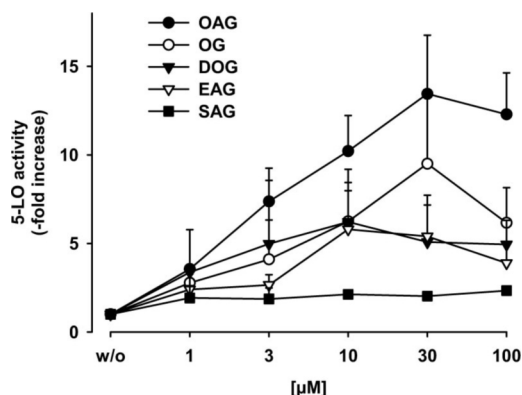


FIG. 3. **Effects of other glycerides on 5-LO activity.** PMNL-S100 were incubated with 1 mM ATP and the indicated glycerides at 1, 3, 10, 30, and 100 μM in PBS plus 1 mM EDTA at 4 $^{\circ}\text{C}$ for 5 min. The samples were prewarmed, and 10 μM AA was added. After 10 min, 5-LO activity was determined. Values are given as mean \pm S.E., $n = 3-4$.

by OG, EAG, and DOG was detectable in the presence of Ca^{2+} (not shown).

OAG Renders 5-LO Activity Resistant against GPx—It was found that Ca^{2+} renders 5-LO activity resistant against inhibition by GPx-1 (26). We attempted to investigate whether OAG could mimic the effect of Ca^{2+} , rendering 5-LO activity GPx-resistant. To restore GPx activity in cell-free assays, PMNL-S100 were supplemented with defined amounts of exogenously added GPx-1 (isolated from bovine erythrocytes), where traces of endogenous thiols derived from the intracellular environment of the PMNL serve as the co-substrate for GPx-1 to suppress 5-LO activity (26). Alternatively, PMNL-S100 containing intrinsic GPx (~ 2.8 milliunits GPx/ 10^6 cells (26)) were supplemented with 1 mM GSH to reconstitute the activity of endogenous GPx enzyme by elevating the co-substrate concentration (compare Ref. 26). In agreement with our previous studies (26), in the absence of Ca^{2+} , 5-LO activity was dose-dependently suppressed by either exogenously added GPx-1 at low thiol concentrations (Fig. 4A) or by supplementation of GSH as the co-substrate for endogenous GPx (Fig. 4B). As observed previously (26), varying amounts of 5-LO enzyme in the incubations (derived from 1, 2.5, 5, or 10×10^6 PMNL) did not alter the degree of 5-LO inhibition by a fixed activity of GPx-1. The GPx-mediated 5-LO inhibition could be reversed by the addition of Ca^{2+} , but OAG (30 μM) also counteracted the suppressive effect of reconstituted GPx activity. It should be noted that 20 μM AA was used, and at this substrate concentration, OAG is not capable of up-regulating 5-LO catalysis (compare Fig. 1E). Dose response studies (Fig. 4, C and D) revealed that 3–10 μM OAG are effective to partly reverse 5-LO inhibition because of GPx activity, and 30–100 μM OAG almost completely restored 5-LO activity. By means of a GPx activity assay, it was found that OAG (up to 100 μM) has no direct inhibitory effect on GPx (100 or 300 units/ml). Also, OAG is no source for LOOH, because no oxidation of the peroxide-sensitive dye 2',7'-dichlorofluorescein diacetate, by itself, was detected (not shown). Reversal of GPx-1-mediated 5-LO inhibition was also evident for other glycerides, such as OG, DOG, EAG, and partially also for SAG (Fig. 4, E and F). Again as observed for OAG, these glycerides did not (EAG and SAG) or only slightly (OG and DOG) up-regulate 5-LO activity in the absence of GPx at 20 μM AA but effectively reversed GPx-mediated suppression of 5-LO.

Mutation of the Phospholipid Binding Site within the C2 Domain Abolishes the Effects of OAG on 5-LO—The C2-like domain of 5-LO binds two Ca^{2+} ions, essentially via the residues Asn-43, Asp-44, and Glu-46 within the loop 2, and muta-

tion of these three residues to alanine (loop2 mut-5LO) leads to diminished Ca^{2+} binding and a requirement for higher Ca^{2+} concentrations to stimulate 5-LO activity (19). In addition, the C2-like domain also binds PC and lipid membranes, which is conferred by tryptophan residues (Trp-13, -75, and -102) located in the putative Ca^{2+} binding loops (20, 21), and mutation of Trp-13, -75, and -102 to alanine causes strongly reduced affinity to PC vesicles (21).

It appeared possible that OAG acts via this putative phospholipid binding domain to stimulate 5-LO catalysis. The tryptophan residues Trp-13, -75, and -102 were mutated to alanine, referred to as 3W mut-5LO. In an ongoing study, we found that, in fact, PC is unable to stimulate the 3W mut-5LO under conditions where the activity of wt-5LO was enhanced by PC.² wt-5LO, loop2 mut-5LO, and 3W mut-5LO were expressed in *E. coli*, and stimulation of enzyme activity by OAG was examined. At 5 μM AA, the activity of wt-5LO in S100 (Fig. 5A) or of purified 5-LO enzyme (Fig. 5B) was concentration-dependently stimulated by OAG (up to 10-fold) in the absence of Ca^{2+} , as observed for 5-LO derived from PMNL. In contrast, no such stimulatory effects of OAG (0.1–100 μM) were observed for the 3W mut-5LO, nor for crude enzyme in S100, nor for purified 5-LO. In control experiments, Ca^{2+} substantially increased the enzymatic activity of 3W mut-5LO. Interestingly, the activity of the loop2 mut-5LO was also up-regulated by OAG, showing that the intact phospholipid binding site (rather than the Ca^{2+} binding residues) is required for enzyme stimulation by glycerides.

Moreover, we assessed the ability of OAG to confer 5-LO enzyme resistance against GPx-1. As shown in Fig. 5C, Ca^{2+} (but also OAG) restored GPx-1-suppressed product synthesis of wt-5LO. In contrast, Ca^{2+} could only partially reverse inhibition of 3W mut-5LO, but OAG had no effect at all. Together, OAG seems to mimic the 5-LO-protecting feature of Ca^{2+} against GPx activity and appears to mediate its effects via the C2-like domain, involving tryptophan residues that are also necessary for phospholipid and membrane binding.

DISCUSSION

In a previous report, we have presented that OAG, and to a minor degree also EAG, function as agonists for human isolated PMNL, stimulating 5-LO product formation by a yet undefined mechanism (30). Here we showed that OAG and other glycerides directly influence 5-LO catalysis. Thus, OAG mimics the 5-LO stimulatory effects of Ca^{2+} *in vitro*, *i.e.* it enhances 5-LO catalysis, particularly at low substrate concentrations ($< 20 \mu\text{M}$), and it renders 5-LO catalysis resistant against inhibition by GPx activity. Intriguingly, various phospholipids prevent 5-LO stimulation by OAG, possibly through displacement of OAG at the putative 5-LO (phospho)lipid binding site. Along these lines, disruption also of this (phospho)lipid binding site (by replacing Trp-13, -75, and -102 by Ala, each (21)) reduces 5-LO stimulation by OAG. Hence we conclude, that OAG mediates its actions via the C2-like β -barrel domain by interacting at a putative (phospho)lipid binding site. Recently it was shown that the OAG-related sphingolipids C_2 -ceramide or ceramide 1-phosphate can interact directly with the C2 domain of the Ca^{2+} -regulated cytosolic phospholipase A_2 , which leads to an increase in enzyme activity (35, 36).

The regulation of 5-LO catalysis is complex and incompletely understood. Stimulating factors *in vitro* include Ca^{2+} , phospholipids or cellular membranes, ATP, LOOH, and yet undefined leukocyte proteins (1). In intact cells, the membrane-bound 5-lipoxygenase-activating protein (18) and phosphorylation events (10, 11, 37) are additional determinants for 5-LO prod-

² A. Michel, D. Steinhilber, and O. Werz, manuscript in preparation.

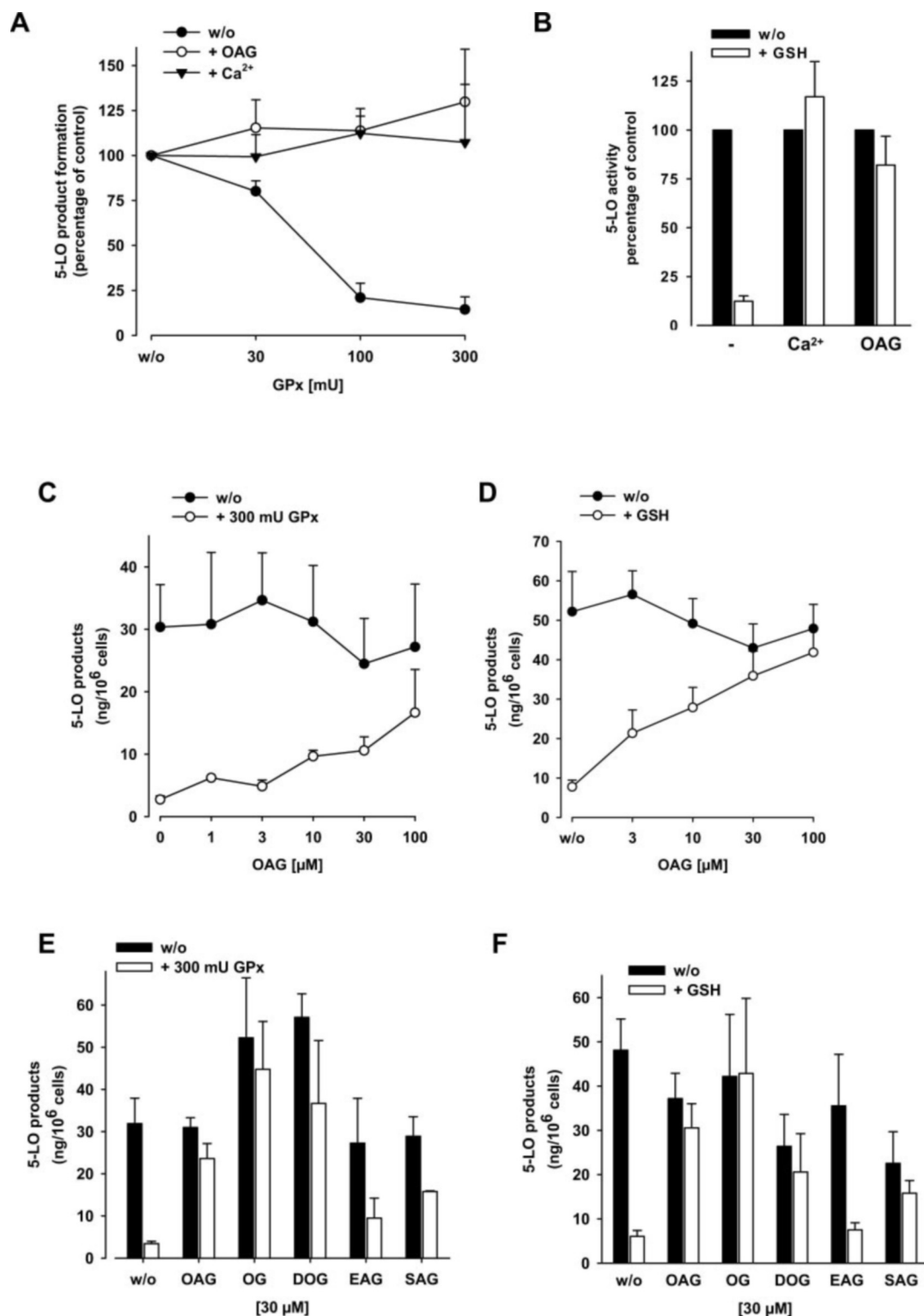
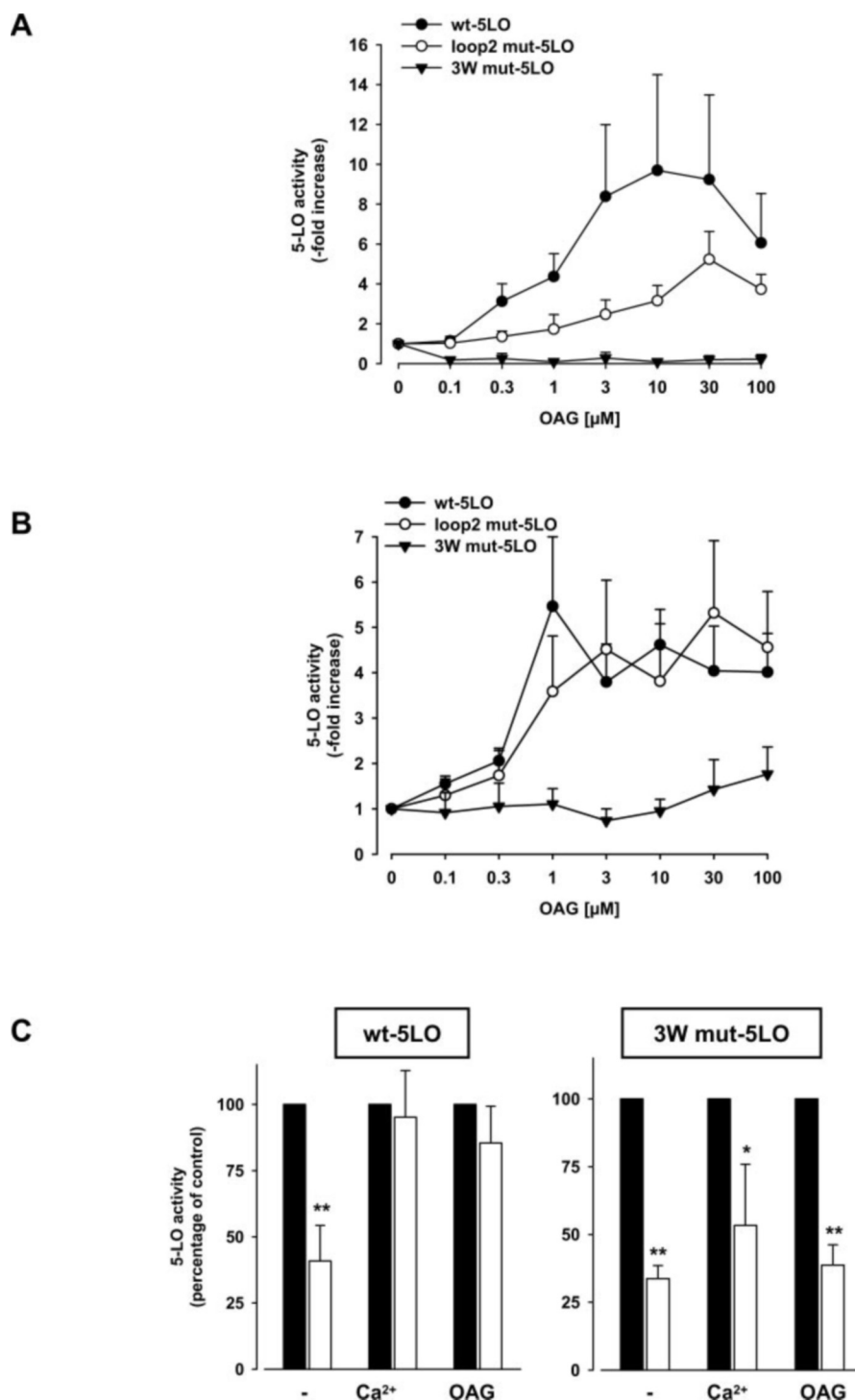


FIG. 4. OAG counteracts GPx-mediated 5-LO inhibition. A, OAG protects 5-LO against GPx-1. To PMNL-S100 diluted in PBS plus 1 mM EDTA and 1 mM ATP, the indicated amounts of GPx-1 (isolated from bovine erythrocytes) were added. The samples were either prewarmed and 20 μ M AA with or without 2 mM CaCl₂ was added or alternatively were preincubated with 10 μ M OAG for 5 min at 4 °C and then prewarmed, and 20 μ M AA was added. After 10 min, 5-LO activity was determined. 5-LO activity in the absence of GPx (control) was 25.9 \pm 12.5, 22.2 \pm 3.8, or 87.1 \pm 16.6 ng/10⁶ cells for S100 incubated without OAG or Ca²⁺, with OAG, or with Ca²⁺, respectively. B, OAG protects 5-LO against GSH. PMNL-S100 were diluted in PBS plus 1 mM EDTA and 1 mM ATP with or without 1 mM GSH. The samples were incubated with or without Ca²⁺ or OAG and 20 μ M AA, as described above, and 5-LO activity was determined. C, PMNL-S100 were diluted in PBS plus 1 mM EDTA, 1 mM ATP with or without GPx-1 (300 milliunits) and OAG, at the indicated concentrations. After 5 min at 4 °C, the samples were prewarmed, 20 μ M AA was added, and 5-LO activity was determined. D, PMNL-S100 were diluted in PBS plus 1 mM EDTA and 1 mM ATP, with or without 1 mM GSH and the indicated concentrations of OAG. After 5 min at 4 °C, the samples were prewarmed, 20 μ M AA was added, and 5-LO activity was determined. E, PMNL-S100 were diluted in PBS plus 1 mM EDTA and 1 mM ATP, with or without GPx-1 (300 milliunits), and the indicated glycerides (30 μ M) were added. After 5 min at 4 °C, the samples were prewarmed, 20 μ M AA was added, and 5-LO activity was determined. F, PMNL-S100 were diluted in PBS plus 1 mM EDTA and 1 mM ATP, with or without 1 mM GSH and the indicated glycerides (30 μ M). After 5 min at 4 °C, the samples were prewarmed, 20 μ M AA was added, and 5-LO activity was determined. Values are given as mean \pm S.E., *n* = 3–4.

FIG. 5. Exchange of the phospholipid binding tryptophan residues (Trp-13, -75, and -102) by alanine prevents stimulation of 5-LO by OAG. wt-5LO, loop2 mut-5LO, and 3W mut-5LO were expressed in *E. coli* S100 containing the 5-LO proteins (A) or partially purified 5-LO proteins (B) were diluted in PBS plus 1 mM EDTA, 1 mM ATP, and the indicated concentrations of OAG. After 5 min at 4 °C, the samples were prewarmed, and 5 μ M AA were added. After 10 min, 5-LO activity was determined. 5-LO activity in the absence of OAG (control) was 16.1 ± 3.5 , 6.25 ± 1.5 , and 4.2 ± 0.8 ng/ml for wt-5LO, loop2 mut-5LO, and 3W mut-5LO, respectively, in S100. For isolated enzymes, the activities of controls were 22.4 ± 2 , 3.2 ± 1.2 , and 2.8 ± 0.8 ng/ml for wt-5LO, loop2 mut-5LO, and 3W mut-5LO, respectively. C, S100 containing wt-5LO (left panel) or 3W mut-5LO (right panel) were diluted in PBS plus 1 mM EDTA and 1 mM ATP, with (white bars) or without (black bars) GPx-1 (200 milliuunits). After 5 min, the samples were prewarmed, and 20 μ M AA were added. Alternatively, OAG (30 μ M) was added, and after 5 min, samples were prewarmed and 20 μ M AA were added. After 10 min, 5-LO activity was determined. Values are given as mean \pm S.E., $n = 3-4$. Student's *t* test: *, $p < 0.05$; **, $p < 0.01$.



uct synthesis, depending on the stimulus, the cell type, and the overall assay conditions. When the mechanism of 5-LO activation by OAG in intact PMNL was investigated, well known pathways, including elevation of $[Ca^{2+}]_i$, involvement of mitogen-activated protein kinase, increased levels of LOOH, or an enhanced accumulation of 5-LO at the nuclear membrane, were excluded (30). This led us to speculate that OAG could directly stimulate 5-LO catalysis.

In agreement with others (27, 28), OAG was unable to augment 5-LO product synthesis in an optimized *in vitro* activity assay (where all stimulatory co-factors were present), regardless of the purity of the enzyme. However, in the absence of Ca^{2+} , OAG strongly enhanced 5-LO activity at the physiologically relevant concentrations (0.3–30 μ M) that are usually ap-

plied to investigate high affinity receptors for DAGs (38). As found for Ca^{2+} (Ref. 23 and Fig. 1D), the stimulatory effect of OAG was evident mainly at AA substrate concentrations of $<20 \mu$ M. In this respect, it is of interest that Ca^{2+} lowers the K_m value for the enzymatic conversion of AA (24, 39). Accordingly, the elevated 5-LO product synthesis by OAG or Ca^{2+} might be due to an increased affinity of the enzyme to its substrate.

It was suggested that Ca^{2+} could facilitate the conversion of the active site iron in 5-LO from the inactive ferrous to the active ferric state by increasing the affinity of the enzyme for LOOH at a putative regulatory fatty acid binding site (26). For OAG, a similar mechanism is conceivable. Thus, OAG reduced the requirement of 5-LO for activating LOOH, visualized by its ability to render 5-LO activity resistant against exogenous as

well as endogenous GPx activity under different experimental settings (Fig. 4, A and B). Such reducing conditions, where GPx activity determines 5-LO activity, actually reflects the situation inside the cell accompanied with a low peroxide tone. Importantly, OAG (as Ca^{2+}) did not inhibit GPx-1 in an *in vitro* assay, excluding the possibility that OAG allows 5-LO product synthesis simply by suppressing GPx activity. Together, it appears that OAG has a similar effect on 5-LO as Ca^{2+} , stimulating the enzyme by increasing the affinity of 5-LO for LOOH. In fact, LOOH mimicked the effect of OAG (or Ca^{2+}), as 13(S)-hydroperoxyoctadecadienoic acid reversed GPx-mediated suppression of 5-LO (26), and the addition of 5-HPETE to PMNL-S100 up-regulated 5-LO activity (Fig. 1F).

Despite these similarities, the molecular mechanisms mediating 5-LO activation appear to be different between Ca^{2+} and OAG. First, for 5-LO stimulation by Ca^{2+} , PC and cationic lipids further increase AA conversion (25, 27, 39), whereas for OAG-stimulated 5-LO, phospholipids or membranes prevent the effect of OAG. Note that in the absence of OAG and Ca^{2+} , all phospholipids tested did not block, but rather increased, 5-LO activity *per se*, as observed previously by others (28, 39, 40). Second, the loop2 mut-5LO, lacking the ability to bind Ca^{2+} (19) and to convert AA in the presence of GPx (26), was still activated by OAG. In contrast, mutation of the Trp-13, -75, and -102 residues, which are necessary for efficient binding to PC (21), led to an enzyme that was not stimulated by OAG and not protected against GPx activity, although Ca^{2+} mediated both actions. Also, in contrast to Ca^{2+} , which causes rapid loss of crude 5-LO activity within a few minutes (41), prolonged exposure (60 min) of 5-LO to OAG did not inactivate 5-LO. Finally, we were unable, in our previous study, to detect an increased binding of 5-LO to the nuclear membrane in response to OAG (30), a feature which is typically mediated by Ca^{2+} (42–44). Together, OAG may stimulate 5-LO in two ways. First, by providing a scaffold for 5-LO, allowing it to activate the enzyme at low ($\leq 10 \mu\text{M}$), but not at high, substrate concentrations. Second, OAG stimulates 5-LO by increasing the affinity for LOOH (which is impaired because of the presence of GPx activity), visualized by stimulating 5-LO at $20 \mu\text{M}$ AA, where OAG (in the absence of GPx) has actually no effect.

DAGs, such as OAG, bind to the so-called C1 domains of a number of proteins including PKC isoenzymes, chimaerins, PKD, RasGRPs, Munc13s, and DAG kinase γ . These enzymes also bind and respond to phorbol esters (*e.g.* PMA) (for review, see Ref. 38). The cysteine-rich C1 domains possess zinc finger-like repeats with the motif $\text{HX}_{12}\text{CX}_2\text{CX}_{13/14}\text{CX}_2\text{CX}_4\text{HX}_2\text{CX}_7\text{C}$ that bind two Zn^{2+} ions. Binding of DAGs or phorbol esters at the C1 domain often acts synergistically with Ca^{2+} -dependent phospholipid binding by the C2 domain, causing activation and/or cellular redistribution to different membranes of the respective enzymes. In analogy to PKC, 5-LO undergoes a Ca^{2+} -dependent activation and redistribution to the nucleus, where 5-LO binds to the PC-rich nuclear membrane mediated by the N-terminal C2-like β -barrel domain. However, in contrast to PKC, 5-LO is not stimulated by PMA *in vitro*, and inspection of the 5-LO primary sequence revealed no motifs of a C1 domain. Also, it remains to be investigated whether 5-LO indeed is a high affinity receptor of OAG or DAGs. Nevertheless, our data suggest that OAG interacts at a phospholipid binding site within the C2-like domain, favored by the finding that phospholipids prevent the effects of OAG and that OAG fails to affect the 3W mut-5LO, lacking residues important for PC binding. Although phospholipids and OAG may act at a common site, the effects of OAG are considerably different from that of phospholipids. Thus, phospholipids alone stimulate 5-LO much less efficiently and are not able to render 5-LO

resistant against GPx. Also, phospholipids (but not OAG) stimulate 5-LO in the presence of Ca^{2+} (25, 27, 28, 39, 40).

C2 domains, first identified in PKC (45), are ~ 130 residues in length and possess the ability to bind Ca^{2+} and phospholipids, whereas phospholipid binding is regulated by Ca^{2+} (46). Besides phospholipids, C2 domains can bind other lipophilic molecules, such as retinoic acid and ceramides, which functionally interact with the C2 domains of PKC- α (47) and cytosolic phospholipase A_2 (35, 36), respectively. 5-LO is built of an N-terminal β -barrel domain that shares structural and functional similarity with C2 domains of other enzymes and a catalytic domain where the active site iron resides (19, 22). Indeed, it has been shown that the 5-LO C2-like domain has two important functions: (i) it acts as a Ca^{2+} -dependent membrane anchor allowing the enzyme to co-localize with cytosolic phospholipase A_2 and 5-lipoxygenase-activating protein at the nuclear membrane (20, 21), and (ii) it mediates Ca^{2+} -induced enzyme activation (19), as well as Ca^{2+} -dependent resistance of 5-LO activity against GPx (26). Also, for the closely related 12- and 15-LO, the existence of N-terminal C2-like β -barrel domains are obvious from the primary sequence or structure (19, 48). However, only the C2 domain of 5-LO has been shown to bind Ca^{2+} with high affinity (19, 21, 49), and enzyme catalysis of 12- and 15-LO is much less affected by Ca^{2+} as compared with 5-LO (50, 51). According to their susceptibility for Ca^{2+} , 12- and 15-LO are activated by OAG to a minor extent, and OAG is less efficient in restoring the GPx-suppressed enzyme activity of 12- and 15-LO.

Certainly, OAG is a synthetic DAG analogue, which, to our knowledge, has not been shown to be endogenously released from stimulated cells. However, one should note that there is no strict specificity of 5-LO for OAG, but other diverse glycerides, such as the monoacylglyceride OG and the diacylglyceride DOG, are also able to stimulate 5-LO and to protect 5-LO against GPx inhibition. Interestingly, the long chain fatty acid diacylglycerol SAG occurring in cells does not stimulate 5-LO but partially protects the enzyme against GPx, indicating that separate mechanisms are operative affecting 5-LO. Approximately 50 different DAGs have been detected in intact cells released by various phospholipases, including PLC, PC-PLC, and PLD/phosphatidate phosphohydrolase, upon the activation of PMNL by many G protein-coupled receptor ligand agonists (*e.g.* *N*-formyl-methionyl-leucyl-phenylalanine, zymosan, platelet-activating factor, complement 5a, etc). Stimulation of 5-LO by endogenously released DAGs could be of relevance for the accomplishment of substantial cellular LT synthesis elicited by agonists or various extracellular stimuli that cause only moderate and transient elevations of $[\text{Ca}^{2+}]_i$ (*e.g.* fMLP, zymosan, PAF, C5a etc). A complex pharmacological approach is currently being conducted in our laboratory to evaluate the role of endogenous (diacyl)glycerides in agonist-induced 5-LO product synthesis and to reveal the physiological relevance of 5-LO stimulation by these lipids. In analogy to PKC or cytosolic phospholipase A_2 , interaction of 5-LO with endogenous glycerides may be a mechanism complementing Ca^{2+} -induced enzyme activation.

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