12(*S*)-Hydroperoxy-eicosatetraenoic acid increases arachidonic acid availability in collagen-primed platelets

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Abstract **Lipid hydroperoxides have been reported to regulate cell function and eicosanoid formation. The aim of the present study was to determine the effect of 12(*S*)-hydroperoxy-eicosatetraenoic acid [12(*S*)-HPETE], the platelet 12-lipoxygenase-derived hydroperoxide of arachidonic acid (AA), on the availability of nonesterified AA, which represents a rate-limiting step in the biosynthesis of eicosanoids. The coincubation of human platelets with concentrations of 12(*S*)-HPETE below 50 nM and subthreshold concentrations (STC) of collagen (less than 0.24 μg/ml) significantly enhanced platelet aggregation and the formation of thromboxane B_2, the stable catabolite of the potent aggregating agent thromboxane A_2. In addition, the nonesterified endogenous AA concentration increased by 3-fold. Arachidonoyl-containing molecular species concentrations of 1,2-diacyl-glycero-3-phosphocholine, 1-alkyl-2-acyl-glycero-3-phosphocholine, and 1-alkenyl-2-acyl-glycero-3-phosphoethanolamine decreased specifically in response to 12(*S*)-HPETE, whereas no significant changes were observed within 1,2-diacyl-glycero-3-phosphoethanolamine and 1,2-diacyl-glycero-3-phosphoinositol molecular species. The 12(*S*)-HPETE-induced increase in nonesterified AA was fully prevented by arachidonoyl trifluoromethyl ketone, an inhibitor of cytosolic phospholipase A_2 ($cPLA_2$), and $cPLA_2$ was translocated to membranes and phosphorylated in platelets incubated with 12(*S*)-HPETE. In conclusion, these results indicate that nanomolar concentrations of 12(*S*)-HPETE could play a significant role in controlling the level of endogenous AA and the formation of thromboxane, thereby potentiating platelet function.**—Calzada, C., E. Véricel, B. Mitel, L. Coulon, and M. Lagarde. **12(*S*)-Hydroperoxy-eicosatetraenoic acid increases arachidonic acid availability in collagen-primed platelets. *J. Lipid Res.* 2001.** 42: **1467–1473.**

Supplementary key words cytosolic phospholipase A_2 • hydroperoxides • phospholipid molecular species • platelet aggregation

The involvement of oxygen-derived free radicals in various pathophysiological states such as cancer and atherosclerosis is well established (1, 2). Lipid peroxidation is one of the main events occurring during the free radical-mediated cellular damage and fatty acid hydroperoxides are the primary intermediates of the nonenzymatic lipid peroxidation process (3). They are also intermediate metabolites in the enzymatic peroxidation of arachidonic acid (AA). In blood platelets, for instance, they are formed via the bifunctional prostaglandin endoperoxide H synthase (PGHS), which leads to prostaglandin G_2 formation, and via the 12-lipoxygenase enzyme, which catalyzes the formation of 12-(*S*)-hydroperoxy-eicosatetraenoic acid [12-(*S*)-HPETE] (4). The latter is further reduced to 12-hydroxy-eicosatetraenoic acid (12-HETE) by glutathione peroxidase (5). There is in vitro evidence that lipid peroxides may regulate eicosanoid formation at the molecular level (6). In this regard, a likely involvement of lipid hydroperoxides concerns hyperaggregable platelets from elderly people. An increased formation of lipid peroxides formed either enzymatically via PGHS and 12-lipoxygenase or nonenzymatically, as well as an alteration of the antioxidant status, were observed in such platelets (7). In particular, glutathione peroxidase, the antioxidant enzyme solely capable of reducing lipid hydroperoxides, was decreased (8), which is likely to result in an increased life span of the substrate, 12(*S*)-HPETE. Because the oxygenases require lipid hydroperoxides for their enzymatic activity (9, 10), it is therefore tempting to hypothesize that the relative accumulation of 12(*S*)-HPETE could explain the increased formation of lipid hydroperoxides and could eventually result in platelet hyperaggregability. We have shown (11) that the addition of hydroperoxides, especially 12(*S*)-HPETE (1–2 μM), to platelets that have been primed with a nonaggregating concentration of AA resulted in an increased formation of thromboxane B_2 (TxB_2), one of the end products of the PGHS pathway, and potentiated platelet aggregation. Besides the stimulation of cyclo-oxygenase activity by lipid hydroperoxides, the potentiation of platelet aggregation might result as well from an increased availability of the substrate AA. Levels of free AA are kept low in resting cells and represent a balance between the release of the free fatty acid from membrane phospholip-

Abbreviations: AA, arachidonic acid; $AACOCF_3$, arachidonoyl trifluoromethylketone; BEL, bromoenol lactone; $cPLA_2$, cytosolic phospholipase A_2; 12-HETE, 12-hydroxy-eicosatetraenoic acid; 12(*S*)-HPETE, 12(*S*)-hydroperoxy-eicosatetraenoic acid; 13(*S*)-HPODE, 13(*S*)-hydroperoxy-octadecadienoic acid; MAFP, methyl arachidonyl fluorophosphonate; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PGHS, prostaglandin H synthase; PI, phosphatidylinositol; STC, subthreshold concentration; TxB_2, thromboxane B_2.

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ids by phospholipase and its re-esterification into phospholipids by acyltransferase (12). On the one hand, Zaleska and Wilson (13) have shown that 25 μM arachidonic or linoleic acid hydroperoxides inhibit AA reacylation into synaptosomal phospholipids. Hydrogen peroxide (200 μM) has also been found to inhibit incorporation of AA into macrophage (14) or vascular smooth muscle cell (15) phospholipids. On the other hand, some studies have reported that hydrogen peroxide or *tert*-butyl hydroperoxide enhanced the release of AA from membrane phospholipids in bovine aortic (16) or bovine pulmonary artery (17) endothelial cells, respectively. However, high concentrations of peroxides have been used in most studies and cannot be considered of physiological relevance. On the basis of these considerations, the present study was undertaken to determine whether low concentrations of 12(*S*)-HPETE prime the platelet response to subaggregating concentrations of collagen, a physiological agonist whose mechanism of action includes the entire AA cascade (18). The effect of 12(*S*)-HPETE on the amount of nonesterified AA as well as the concentration of the AA-containing molecular species from phospholipid subclasses were determined. To obtain evidence of the involvement of the 85-kDa Ca^{2+}-dependent cytosolic phospholipase A_2 ($cPLA_2$, type IV) in the mechanism of action of 12(*S*)-HPETE, arachidonoyl trifluoromethylketone ($AACOCF_3$), an inhibitor of $cPLA_2$, was tested and the translocation of the enzyme from the cytosol to membranes as well as its phosphorylation were studied as it represents two critical steps in its regulation (19).

MATERIALS AND METHODS

Materials

12(*S*)-HPETE, 15(*S*)-hydroperoxy-eicosatetraenoic acid [15(*S*)-HPETE], 13(*S*)-hydroperoxy-octadecadienoic acid [13(*S*)-HPODE], aristolochic acid, and $AACOCF_3$ were purchased from Biomol Research Laboratories (Plymouth Meeting, PA) and were 98% pure; ethanolic solutions of hydroperoxides were purged with nitrogen and stored at −80°C until use. Bromoenol lactone (BEL) and methyl arachidonyl fluorophosphonate (MAFP) were from Cayman (Ann Arbor, MI). [^{3}H]AA (210 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Mouse monoclonal antibody directed against $cPLA_2$ (4-4B-3C) was from Santa Cruz Biotechnology (Santa Cruz, CA) and goat anti-mouse IgG(H+L) horseradish peroxidase conjugate was from Bio-Rad (Hercules, CA). Solvents were from SDS (Peypin, France) and silica gel 60 plates were purchased from Merck (Darmstadt, Germany). All other reagents were purchased from Sigma (St. Louis, MO).

Platelet isolation

Blood was collected at the local blood bank from healthy volunteers who had not ingested any drugs interfering with platelet functions in the previous 10 days. Blood was drawn into a one-seventh volume of CPD (19.6 mM citric acid, 89.4 mM sodium citrate, 16.1 mM NaH_2PO_4, 128.7 mM dextrose; pH 5.6). Platelets were isolated by a previously described method (20). Briefly, blood was centrifuged at 200 *g* for 15 min at 20°C to obtain platelet-rich plasma (PRP). PRP was acidified to pH 6.4 with 0.15 M citric acid and immediately centrifuged at 900 *g* for 10 min at 20°C. Pelleted platelets were resuspended into a Tyrode-HEPES buffer solution (137 mM NaCl, 2.7 mM KCl, 11.9 mM $NaHCO_3$, 0.41 mM NaH_2PO_4, 1 mM $MgCl_2$, 5.5 mM glucose, 5 mM HEPES; pH 7.35). Platelet suspensions were left for 1 h at room temperature before experiments were started.

Platelet aggregation

Platelet suspensions were preincubated for 2 min at 37°C, and then incubated in the presence or absence of HPETE for 1 min at 37°C before the addition of a subthreshold concentration (STC) of collagen for another 4 min with continuous stirring at 1,000 rpm. Platelet aggregation was measured in a Chronolog dual channel aggregometer (Coulter, Margency, France) according to the turbidimetric method of Born (21). The STC of collagen was defined as the highest concentration of collagen that induced less than an 8% increase in light transmission, the light transmission through Tyrode-HEPES solution representing 100% aggregation. After incubation of platelets with an STC of collagen in the presence or absence of 12(*S*)-HPETE, platelets were immediately centrifuged at 5,000 *g* for 5 min and supernatants were stored until assayed. The concentration of TxB_2, the stable catabolite of unstable thromboxane A_2 (TxA_2), was determined by enzyme immunoassay (Cascade Biochem, Reading, UK).

Quantification of nonesterified AA level

Platelet suspensions were preincubated for 2 min at 37°C, and then incubated in the presence or absence of HPETE for 1 min at 37°C before the addition of an STC of collagen for another 1 min with continuous stirring at 1,000 rpm. After incubation of platelet suspension (1 volume), 3 volumes of ethanol was added in the presence of heptadecanoic acid as an internal standard. Platelet lipids were extracted twice with chloroform (6 volumes) in the presence of 50 μM butylated hydroxytoluene as an antioxidant. Lipid classes were separated by thin-layer chromatography (TLC) on silica gel 60G plates with the solvent mixture hexane–diethyl ether–acetic acid 80:20:1 (v/v/v) into phospholipids, nonesterified fatty acids, and neutral lipids (22). The nonesterified fatty acid-containing zone was scraped off the silica gel, extracted three times with ether–methanol 9:1 (v/v), and derivatized with diazomethane (23). Fatty acid methyl esters were separated by gas-liquid chromatography, using a Delsi chromatograph model DI 200 equipped with an SP2380 capillary column (30 m × 0.32 mm; Supelco, Bellefonte, PA) and quantified according to the amount of internal standard.

Quantification of molecular species in phospholipid subclasses

Platelet suspensions were first labeled with [^{3}H]AA (1 μCi/ml, 5 nM) for 1 h at room temperature and then incubated as described above. Internal standards, namely 1,2-diheptadecanoyl-*sn*-glycero-3-phosphocholine, 1-*O*-hexadecyl-2-hexadecanoyl-glycero-3-phosphocholine, and 1,2-diheptadecanoyl-*sn*-glycero-3-phosphoethanolamine, were added to platelets. After lipid extraction, chloroform–methanol 80:8 (v/v) was used as an eluent to separate by TLC phospholipids from nonesterified and hydroxylated fatty acids, triacylglycerols, and cholesterol. Phospholipid classes were then resolved by TLC with the developing solvent chloroform–methanol–methylamine 60:20:5 (v/v/v) into phosphatidylinositol (PI) (R_f = 0.09), phosphatidylserine (PS) (R_f = 0.20), phosphatidylcholine (PC) (R_f = 0.48), and phosphatidylethanolamine (PE) (R_f = 0.66) (24). Each phospholipid class was extracted from the silica gel with chloroform–methanol–water 5:5:1 (v/v/v) and hydrolyzed by phospholipase C (from *Bacillus cereus*) according to the method of Takamura et al. (25) modified by Croset, Bayon, and Lagarde (26). The resulting diradylglycerols were treated with 3,5-dinitrobenzoyl chloride in dry pyri-

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dine for 45 min at 60°C and dinitrobenzoyl derivatives were extracted twice with hexane after being cooled and the addition of water. Subclasses of diradylglyceronitrobenzoates were separated by TLC with the solvent mixture toluene–hexane–diethyl ether 50:45:4 (v/v/v) into 1,2-diacyl (R_f = 0.20), 1-alkyl-2-acyl (R_f = 0.40), and 1-alkenyl-2-acyl (R_f = 0.46), which were identified with a Berthold (Bad Wildbad, Germany) TLC linear analyzer. Each phospholipid subclass was extracted from the silica gel twice with hexane–diethyl ether 1:1 (v/v). Various molecular species in phospholipid subclasses were separated by HPLC. A Superspher 100 RP-18 column (5 μm, 4.6 mm i.d. × 250 mm) was used with the solvent system acetonitrile–2-propanol 9:1 (v/v) at a flow rate of 1.5 ml/min. The radioactive species were visualized with a Berthold HPLC radioactivity monitor and the separated molecular species were quantified by absorbance at 240 nm according to the amount of relevant internal standard.

SDS-PAGE and immunoblotting

Platelets were preincubated for 2 min at 37°C and then incubated in the presence or absence of 12(*S*)-HPETE (100 nM) or 12-HETE (100 nM) for 2 min at 37°C. After incubation, platelet suspensions were immediately centrifuged at 700 *g* for 10 min and the resultant pellet was resuspended in ice-cold lysis buffer containing protease and phosphatase inhibitors as described by Kramer et al. (27). After sonication, lysates were centrifuged at 100,000 *g* for 1 h at 4°C to obtain cytosolic and membrane fractions. Protein concentration was determined by the Bradford assay (28). The proteins (50 μg) were denatured for 15 min at 60°C, electrophoresed in 7.5% Tris-HCl polyacrylamide gels at 25 mA for 2 h and 30 min, and transferred to nitrocellulose membranes (60 V, 1 h). The membranes were then blocked in 10% fat-free dried milk, 0.1% Tween 20, Tris-buffered saline, incubated with anti-cPLA$_2$ monoclonal antibody (dilution, 1:1,000) for 2 h, washed, and incubated with 1:2,000 goat anti-mouse IgG(H+L) horseradish peroxidase conjugate for 2 h. cPLA$_2$ was visualized by using the enhanced chemiluminescence detection system and the density of the relevant band was analyzed with a video densitometer (Bioprofil; Vilber Lourmat, Marne la Vallée, France).

Analysis of results

Results are expressed as means ± SEM. Differences between means were assessed by the Student's paired *t*-test. Statistical significance was assessed with $P < 0.05$.

RESULTS

Effect of 12(*S*)-HPETE on platelet aggregation and nonesterified AA concentration

As shown in **Table 1**, the preincubation of platelets with nanomolar concentrations of 12(*S*)-HPETE (below 50 nM) for 1 min at 37°C significantly induced the aggregation of platelets further incubated with an STC of collagen. Although the effective concentrations of 12(*S*)-HPETE varied from one experiment to another, they ranged from 20 to 100 nM. The replacement of 12(*S*)-HPETE by its hydroxylated derivative 12-HETE did not potentiate the platelet response to the agonist, nor did 12(*S*)-HPETE alone have an effect on platelet aggregation (data not shown). Under the same experimental conditions, other lipoxygenase-derived hydroperoxy fatty acids, 15(*S*)-HPETE and 13(*S*)-HPODE, also primed the platelet response to STC of collagen but were less potent than

TABLE 1. Effect of 12-hydroperoxy-eicosatetraenoic acid on platelet aggregation and nonesterified arachidonic acid level

	Nonesterified AA	Aggregation
	pmol/10⁹ platelets	%
Collagen	87 ± 21	6 ± 1
12(*S*)-HPETE + collagen	273 ± 77[a]	56 ± 7[b]
AACOCF$_3$ + collagen	60 ± 9	2 ± 1
AACOCF$_3$ + 12(*S*)-HPETE + collagen	50 ± 18[c]	4 ± 1[c]

Platelets were preincubated in the absence or presence of 50 μM AACOCF$_3$ for 2 min at 37°C. They were then incubated in the absence or presence of 12(*S*)-HPETE (47 ± 9 nM) for 1 min at 37°C followed by the addition of a subthreshold concentration of collagen (0.24 ± 0.03 μg/ml). The extent of platelet aggregation was determined 4 min after the addition of the agonist. The incubation was stopped 1 min after the addition of collagen for the quantification of nonesterified AA level. After lipid extraction and separation of nonesterified fatty acids by TLC, nonesterified AA was quantified by gas chromatography as described in Materials and Methods. Results are expressed as means ± SEM of five to nine experiments.

[a] $P < 0.01$, compared with platelets incubated solely with collagen.

[b] $P < 0.001$, compared with platelets incubated solely with collagen.

[c] $P < 0.05$, compared with platelets coincubated with 12(*S*)-HPETE and collagen.

12(*S*)-HPETE [50 nM 15(*S*)-HPETE and 50 nM 13(*S*)-HPODE induced, respectively, 32 ± 18% and 37 ± 13% platelet aggregation whereas 50 nM 12(*S*)-HPETE induced 52 ± 19% platelet aggregation, mean ± SD, n = 3]. The priming effect of 12(*S*)-HPETE on platelet aggregation was also associated with an increased formation of TxB$_2$, the stable catabolite of the potent aggregating agent, TxA$_2$. For instance, the addition of 50 nM 12(*S*)-HPETE to platelets incubated with nonaggregating concentrations of collagen resulted in a 4-fold increase of TxB$_2$ formation as compared with its formation in platelets incubated with an STC of collagen (414 ± 70 vs. 106 ± 26 pmol/10^9 platelets, mean ± SEM, n = 4, $P < 0.05$). The effect of 12(*S*)-HPETE on the eicosanoid precursor AA was then investigated by quantifying nonesterified AA concentration by gas chromatography rather than by estimating it by determining [^{3}H]AA release. The incubation of platelets with an STC of collagen in the absence or presence of 12(*S*)-HPETE was stopped 1 min after the addition of the agonist to minimize the conversion of released AA to derived oxygenated metabolites. As shown in Table 1, the concentration of nonesterified AA in platelets incubated with an STC of collagen (0.24 ± 0.03 μg/ml) was very low (87 ± 21 pmol/10^9 platelets). The addition of 12(*S*)-HPETE (47 ± 9 nM) to platelets incubated with an STC of collagen (0.24 ± 0.03 μg/ml) resulted in a 3-fold increase in the amount of nonesterified AA, which was associated with a significant increase in platelet aggregation. Moreover, the addition of 12(*S*)-HPETE alone to control platelets had no effect on the amount of nonesterified AA (95 vs. 103 pmol/10^9 platelets plus 78 nM 12(*S*)-HPETE, n = 2). To investigate whether the 12(*S*)-HPETE-induced AA rise could be due to the activation of cPLA$_2$, an enzyme involved in the receptor-mediated release of AA from membrane phospholipids (27), platelet suspensions were preincubated with an inhibitor of cPLA$_2$, AACOCF$_3$ (29), before the addition of 12(*S*)-HPETE and an STC of collagen. First, it

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was checked that the addition of 50 μM $AACOCF_3$ to platelets incubated solely with an STC of collagen had no significant effect on the amount of nonesterified AA in platelets as compared with control platelets (Table 1). Interestingly, the preincubation of platelets with $AACOCF_3$ fully prevented the increase in nonesterified AA concentration induced by 12(*S*)-HPETE as well as the enhanced platelet aggregation. In the same way, 10 μM MAFP, an inhibitor of $cPLA_2$ (30), or 100 μM aristolochic acid, an inhibitor of Ca^{2+}-dependent PLA_2 (31), prevented the increased formation of TxB_2 as well as the enhanced aggregation in platelets coincubated with 12(*S*)-HPETE and an STC of collagen. These results suggest an activation of $cPLA_2$ in response to 12(*S*)-HPETE. However, because $AACOCF_3$ and MAFP also inhibit Ca^{2+}-independent phospholipase A_2 ($iPLA_2$), the effect of BEL, a rather specific inhibitor of $iPLA_2$ (32), on TxB_2 formation was tested. Surprisingly, 10 μM BEL prevented 12(*S*)-HPETE-induced platelet aggregation and increased TxB_2 formation, suggesting that $iPLA_2$ may contribute to 12(*S*)-HPETE-induced AA release.

Effect of 12(*S*)-HPETE on molecular species within phospholipid subclasses

To thoroughly characterize membrane phospholipids, the endogenous concentrations of molecular species within phospholipid subclasses were determined by HPLC. As shown in **Table 2**, in the 1,2-diacyl-glycero-3-phosphocholine (GPC) subclass, a significantly decreased concentration of the two main AA-containing molecular species, 16:0–20:4 and 18:0–20:4, occurred in platelets coincubated with 12(*S*)-HPETE and collagen (−20% and −17%, respectively) whereas the minor 18:1–20:4 species did not decrease significantly. A 12% decrease in one or more of the molecular species 16:0–18:2, 18:1–22:4, and 16:0–16:1 (present in one HPLC peak) was observed in 12(*S*)-HPETE-treated platelets and likely reflected a decrease in the molecular species 16:0–18:2, which contains the fatty acids more represented in PC (33). However, neither the 18:0–18:2, 16:0–18:1, and 18:1–18:1 species nor the 16:0–16:0 and 18:0–18:1 species significantly decreased in platelets coincubated with 12(*S*)-HPETE and collagen. In the 1-alkyl-2-acyl-GPC subclass, the AA-containing species were predominant among molecular species and both 16:0–20:4 and 18:0–20:4 species decreased significantly in response to 12(*S*)-HPETE [1.2 ± 0.3 and 1.0 ± 0.2 nmol/10^9 platelets treated with collagen at 0.17 μg/ml vs. 0.9 ± 0.2 and 0.7 ± 0.2 nmol/10^9 platelets coincubated with 21 nM 12(*S*)-HPETE and collagen at 0.17 μg/ml, respectively, n = 8, $P < 0.05$]. The 1,2-diacyl-glycero-3-phosphoethanolamine (GPE) subclass was especially rich in the molecular species 18:0–20:4 and 16:0–20:4 as already described (26) (Table 2). However, none of the molecular species concentrations was significantly affected by the treatment of platelets with 12(*S*)-HPETE. By contrast, 18:1–20:4 and 16:0–20:4 species of the 1-alkenyl-2-acyl-glycero-3-phosphoinositol subclass decreased significantly (−46% and −33%, respectively) and the molecular species 18:0–20:4 tended to decrease in 12(*S*)-HPETE-treated platelets (−24%). Finally, the concentration of 18:0–20:4 species, the main one within the 1,2-diacyl-glycero-3-phosphoinositol subclass, was unchanged and even tended to increase in 12(*S*)-HPETE-treated platelets [6.4 ± 1.9 nmol/10^9 platelets treated with collagen at 0.18 μg/ml vs. 9.3 ± 1.9 nmol/10^9 platelets coincubated with 18 nM 12(*S*)-HPETE and collagen at 0.18 μg/ml, n = 5].

Effect of 12(*S*)-HPETE on translocation and phosphorylation of $cPLA_2$

To obtain biochemical evidence that $cPLA_2$ was indeed involved in the 12(*S*)-HPETE-mediated effect, SDS-PAGE/immunoblotting analysis of platelet cytosolic and mem-

TABLE 2. Effect of 12-hydroperoxy-eicosatetraenoic acid on molecular species content of phosphatidylcholine and phosphatidylethanolamine subclasses

Molecular Species	1,2-Diacyl-GPC: Platelets + Collagen	1,2-Diacyl-GPC: Platelets + 12(*S*)-HPETE + Collagen	1,2-Diacyl-GPE: Platelets + Collagen	1,2-Diacyl-GPE: Platelets + 12(*S*)-HPETE + Collagen	1-Alkenyl-2-acyl-GPE: Platelets + Collagen	1-Alkenyl-2-acyl-GPE: Platelets + 12(*S*)-HPETE + Collagen
	nmol/10^9 platelets					
18:1–20:4	1.5 ± 0.3	1.3 ± 0.3	2.5 ± 0.6	2.7 ± 0.5	1.1 ± 0.2	0.6 ± 0.1[a]
16:0–20:4	12.5 ± 1.6	10.1 ± 1.1[b]	7.9 ± 2.3	7.8 ± 1.9	13.5 ± 3.1	9.1 ± 2.6[b]
18:0–20:4	13.4 ± 1.6	11.1 ± 1.2[a]	40.1 ± 9.1	37.6 ± 8.2	20.2 ± 3.2	15.3 ± 3.9
16:0–18:2 + 18:1–22:4 + 16:0–16:1	22.8 ± 2.4	20.1 ± 2.4[b]	4.3 ± 1.4	3.4 ± 1.1	—	—
18:0–18:2 + 16:0–18:1 + 18:1–18:1	51.0 ± 5.7	46.5 ± 5.3	6.8 ± 5.8	5.9 ± 1.8	—	—
16:0–16:0	9.4 ± 1.0	8.8 ± 1.0	1.2 ± 0.3	1.2 ± 0.3	—	—
18:0–18:1	15.5 ± 1.8	14.6 ± 1.9	1.6 ± 0.3	1.5 ± 0.4	—	—

Isolated human platelets were incubated for 1 min at 37°C in the absence or presence of 12(*S*)-HPETE (21.0 ± 3.6 nM) followed by the addition of an STC of collagen (0.18 ± 0.02 μg/ml) for another 1 min. After lipid extraction, separation of phospholipid classes by TLC, hydrolysis by phospholipase C and treatment with dinitrobenzoyl chloride, dinitrobenzoyl derivatives of diradylglycerols were separated by TLC and individual molecular species were separated by HPLC on a Superspher 100 RP-18 column and quantified by absorbance at 240 nm. Platelet aggregation tests were performed in parallel for each experiment. Collagen alone induced 7.7 ± 1.2% aggregation whereas 12(*S*)-HPETE and collagen induced 59.9 ± 5.8% aggregation. Values represent means ± SEM of five to eight different experiments. GPC, glycero-3-phosphocholine; GPE, glycero-3-phosphoethanolamine.

[a] $P < 0.01$, compared with platelets incubated solely with collagen.

[b] $P < 0.05$, compared with platelets incubated solely with collagen.

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brane fractions was carried out with anti-cPLA$_2$ antibody. The translocation of cPLA$_2$ from the cytosol to membranes was investigated as it constitutes a critical step in the regulation of the enzyme. Platelets were preincubated for 2 min at 37°C and incubated in the absence or presence of 12(*S*)-HPETE (100 nM) or 12-HETE (100 nM) for 2 min at 37°C. The amount of cPLA$_2$ significantly increased in membranes of platelets incubated with 100 nM 12(*S*)-HPETE as compared with control platelets (129 ± 6% vs. 100%, $P < 0.01$, n = 7). On the contrary, no change in the amount of membrane-associated cPLA$_2$ was observed in 12-HETE-treated platelets (94 ± 10%), ruling out a putative involvement of the reduced form of 12(*S*)-HPETE. There was no significant change in the quantity of cPLA$_2$ in cytosolic fractions of platelets incubated either in the absence or presence of 12(*S*)-HPETE or 12-HETE. To determine whether cPLA$_2$ is phosphorylated on 12(*S*)-HPETE stimulation, the electrophoretic mobility of cPLA$_2$ was determined in control and 12(*S*)-HPETE-treated platelets because the phosphorylation of the enzyme produces a characteristic retardation in migration of this protein when analyzed by SDS-PAGE. As shown in **Fig. 1**, the incubation of platelets with 12(*S*)-HPETE for 2 min resulted in a shift to the slower migrating form (phosphorylated cPLA$_2$) in SDS-PAGE whereas the faster migrating form (unphosphorylated cPLA$_2$) was the prominent form in control platelets. Therefore, 12(*S*)-HPETE caused the translocation of cPLA$_2$ to membranes as well as its phosphorylation.

DISCUSSION

There is increasing evidence that lipid hydroperoxides modulate cell functions. Whereas the inhibitory effect of relatively high concentrations of HPETE on platelet aggregation has been described (34, 35), more recent studies have shown that lower concentrations of H_2O_2 (36) or HPETE (11) can prime the aggregation of platelets in response to nonaggregating concentrations of AA via stimulation of cyclo-oxygenase activity. The results of the present study show that even lower concentrations of 12(*S*)-HPETE potentiate the aggregation of platelets coincubated with nonaggregating concentrations of collagen, an agonist acting through the release of AA from membrane phospholipids. It is noteworthy that the priming effect of 12(*S*)-HPETE on the platelet response to collagen was observed at concentrations below 50 nM. Such amounts can be considered of physiological relevance as basal concentrations of free monohydroxylated fatty acids in human resting platelets have been evaluated to 1.5 pmol/10^9 platelets, which corresponds to 100 nM, 12-HETE representing 50 nM within total monohydroxylated fatty acids (37). Our results also show that nanomolar concentrations of 12(*S*)-HPETE increased the amount of nonesterified AA in platelets further exposed to an STC of collagen. It is therefore likely that such a hydroperoxide-induced AA rise resulted in an increased formation of the proaggregatory AA metabolite TxA$_2$, and subsequently in enhanced platelet aggregation, as shown. Previous studies have reported a stimulation of AA release by peroxides (16, 17), correlating with an increased serine phosphorylation of cPLA$_2$ (38), but the concentrations used were relatively high (at least 100 μM hydrogen peroxide or *tert*-butyl hydroperoxide). Because AA is mainly released from the *sn*-2 position of membrane phospholipids by the action of an 85-kDa calcium-sensitive cPLA$_2$ on activation of platelets with physiological agonists, such as collagen and thrombin (19), its involvement in the mechanism of action of 12(*S*)-HPETE was investigated. Our results indicate that the 12(*S*)-HPETE-induced increase in nonesterified AA showed some characteristics that would be expected if it were indeed mediated by cPLA$_2$. First, the coincubation of platelets with an STC of collagen and 12(*S*)-HPETE induced a preferential decrease in AA from the molecular species of 1,2-diacyl-GPC, 1-alkyl-2-acyl-GPC, and 1-alkenyl-2-acyl-GPE. Although other molecular species containing unsaturated fatty acids at the *sn*-2 position decreased within 1,2-diacyl-GPC in platelets stimulated with 12(*S*)-HPETE, they represent lower quantities compared with AA-containing molecular species. Concerning the type of polar head group at the *sn*-3 position of phospholipids, PC and PE were preferentially hydrolyzed in platelets coincubated with 12(*S*)-HPETE and an STC of collagen whereas PI remained unchanged. Previous studies using a mixed vesicle model have shown that the 85-kDa cPLA$_2$ prefers PC slightly over PE and PI (39) but in platelets stimulated with thrombin, PC and PE were found to be the main sources of AA (40). Within PC subclasses, there was no preference for the radyl group at the *sn*-1 position, both 1,2-diacyl-GPC and 1-alkyl-2-acyl-GPC subclasses being hydrolyzed in the presence of 12(*S*)-HPETE. According to Diez et al. (41), cPLA$_2$ showed no difference between PC substrates containing either acyl or alkyl linkage at the *sn*-1 position. Interestingly, within PE subclasses, AA decreased selectively in 1-alkenyl-2-acyl-GPE over 1,2-diacyl-GPE in platelets treated with 12(*S*)-HPETE. This result corroborates a study showing that oxidation of bovine brain PE by hydrogen peroxide and copper resulted in a loss of specific plasmalogen molecular species containing polyunsaturated fatty acids at the *sn*-2 position whereas diacyl-GPE species were much more

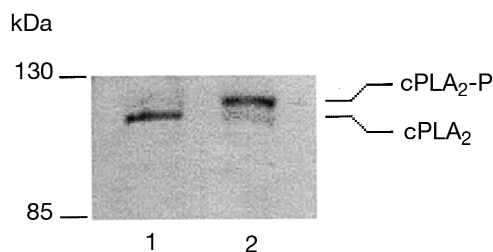

Fig. 1. Effect of 12(*S*)-HPETE on cPLA$_2$ phosphorylation. Platelets were preincubated for 2 min at 37°C and incubated in the absence or presence of 12(*S*)-HPETE (100 nM) for 2 min at 37°C. The lysates (50 μg) were electrophoresed on 7.5% gels and transferred to nitrocellulose membranes. Immunoblottings were performed with mouse monoclonal antibodies raised against cPLA$_2$. Lane 1, control platelets; lane 2, platelets incubated with 100 nM 12(*S*)-HPETE. The data presented are representative of three experiments.

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stable (42). A second piece of evidence in favor of 12(*S*)-HPETE-induced $cPLA_2$ activation is the prevention of increased AA content by the $cPLA_2$ inhibitor $AACOCF_3$, which is selective for $cPLA_2$ among known Ca^{2+}-dependent enzymes (29). Finally, direct evidence of activation of $cPLA_2$ by 12(*S*)-HPETE was presented by considering the translocation of the enzyme from the cytosol to membranes and its phosphorylation. In platelets incubated with 12(*S*)-HPETE, the quantity of $cPLA_2$ increased in membranes, which may be responsible in part for AA release from phospholipids, and $cPLA_2$ underwent a shift in electrophoretic mobility, indicative of $cPLA_2$ phosphorylation on Ser-505 (43). Altogether, our results are in favor of the involvement of $cPLA_2$ in the mechanism of action of 12(*S*)-HPETE. However, $iPLA_2$ seems to contribute to the 12(*S*)-HPETE-induced AA liberation because BEL prevented the increased formation of TxB_2, one of the major metabolites of AA. The role of $iPLA_2$ in phospholipid remodeling and signal transduction remains to be defined in platelets.

In conclusion, 12(*S*)-HPETE may have an important role in controlling the level of nonesterified AA, which constitutes a rate-limiting step in the biosynthesis of biologically active eicosanoids.

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