Low concentrations of lipid hydroperoxides prime human platelet aggregation specifically via cyclo-oxygenase activation

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There is mounting evidence that lipid peroxides contribute to pathophysiological processes and can modulate cellular functions. The aim of the present study was to investigate the effects of lipid hydroperoxides on platelet aggregation and arachidonic acid (AA) metabolism. Human platelets, isolated from plasma, were incubated with subthreshold (i.e. non-aggregating) concentrations of AA in the absence or presence of hydroperoxyeicosatetraenoic acids (HPETEs). Although HPETEs alone had no effect on platelet function, HPETEs induced the aggregation of platelets co-incubated with non-aggregating concentrations of AA, HPETEs being more potent

than non-eicosanoid peroxides. The priming effect of HPETEs on platelet aggregation was associated with an increased formation of cyclo-oxygenase metabolites, in particular thromboxane A_2 , and was abolished by aspirin, suggesting an activation of cyclo-oxygenase by HPETEs. It was not receptor-mediated because the 12-HPETE-induced enhancement of AA metabolism was sustained in the presence of SQ29,548 or RGDS, which blocked the aggregation. These results indicate that physiologically relevant concentrations of HPETEs potentiate platelet aggregation, which appears to be mediated via a stimulation of cyclo-oxygenase activity.

INTRODUCTION

Blood platelets have a vital role in haemostatic processes and in pathological events such as complications of thrombosis and atherosclerosis [1]. After vessel injury, one of the earliest events is the adhesion of circulating platelets to the sub-endothelium followed by platelet aggregation and secretion of the granule contents. During platelet activation, arachidonic acid (AA) is released from membrane phospholipids and oxygenated by prostaglandin endoperoxide synthase (PGHS) and 12-lipoxygenase. PGHS catalyses both the oxygenation of AA to prostaglandin (PG) G_2 via its cyclo-oxygenase activity and the subsequent reduction of PGG_2 to PGH_2 via its peroxidase activity [2]. PGH_2 is further metabolized to thromboxane A_2 (TXA_2), a very potent aggregatory agent, to 12-hydroxyheptadecatrienoic acid (12-HHT) plus malondialdehyde and also to prostaglandins. Platelet 12-lipoxygenase acts on AA to form 12-hydroperoxyeicosatetraenoic acid (12-HPETE), further reduced to 12-hydroxyeicosatetraenoic acid (12-HETE) by a glutathione-peroxidase [3]. The platelet hyperactivation observed in elderly people and diabetic patients is associated with an increased formation of oxygenated AA metabolites and a decreased antioxidant status [4,5]. In particular, a lower activity of glutathione-peroxidase has been reported in aging [6], which might result in a transient accumulation of lipid hydroperoxides. It is conceivable that an increased life span of AA-derived hydroperoxides might stimulate the oxygenase activities because they require peroxides to be active [7], which might result in platelet hyperactivation. It has been established that low concentrations of lipid peroxides activate cyclo-oxygenase activity [8] but high concentrations are also inhibitory [9]. However, the biological functions of lipoxygenase metabolites of AA are still

not well characterized. Most studies performed in platelets incubated with relatively high concentrations of hydroperoxides have reported an inhibitory effect of these hydroperoxides on platelet aggregation [10,11] but no data to our knowledge have shown a stimulatory effect of lipid hydroperoxides on platelet function. In this context it is of interest to determine whether physiologically relevant concentrations of HPETEs induce platelet aggregation or enhance the platelet response to agonists. The effects of 12-HPETE, a key intermediate of oxidant generation in platelets, were mainly compared with those of 15-HPETE, a positional isomer. The results indicate that low concentrations of HPETEs induce the aggregation of platelets co-incubated with subthreshold concentrations (STCs) of AA. These effects are mainly mediated via a stimulation of the cyclo-oxygenase activity.

EXPERIMENTAL

Materials

12-HPETE and 12-HETE were purchased from Cascade Biochem (Reading, Berks., U.K.) and were 98% pure. 15-HPETE was synthesized from AA by lipoxidase type I-B [12] and 15-HETE was obtained from 15-HPETE after reduction with sodium borohydride. Both hydroperoxides were purged with nitrogen and stored at -70°C until use. $[1-^{14}\text{C}]\text{AA}$ (57 Ci/mol) was obtained from DuPont–New England Nuclear (Boston, MA, U.S.A.). Silica gel 60 plates and solvents were from Merck (Darmstadt, Germany). AA, tert-butyl hydroperoxide (tBH), H_2O_2 , α -tocopherol, desferrioxamine mesylate, lipoxidase type I-B, sodium borohydride and RGDS were all purchased from

Abbreviations used: AA, arachidonic acid; HETE, hydroxyeicosatetraenoic acid; HHT, hydroxyheptadecatrienoic acid; HPETE, hydroperoxyeicosatetraenoic acid; LDL, low-density lipoproteins; PG, prostaglandin; PGHS, prostaglandin endoperoxide synthase; STC, subthreshold concentration; tBH, tert-butyl hydroperoxide; TXA_2 , thromboxane A_2 ; TXB_2 , thromboxane B_2 .

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Sigma (St. Louis, MO, U.S.A.). SQ29,548 was a gift from Dr. M. Ogletree (Squibb Institute for Research, Princeton, NJ, U.S.A.).

Platelet isolation

Blood was drawn from healthy volunteers who had not ingested any drugs interfering with platelet functions in the previous 10 days. Venous blood was collected into 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)] as an anticoagulant and centrifuged at 200 *g* for 15 min at 20 °C to obtain platelet-rich plasma. Platelets were isolated by a previously described method [13]. Briefly, platelet-rich plasma was acidified to pH 6.4 with 0.15 M citric acid and immediately centrifuged at 900 *g* for 10 min at 20 °C. Sedimented 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 aggregation studies were started.

Platelet aggregation

Platelet aggregation was measured in isolated platelets with the turbidimetric method of Born [14] in a Chrono-log dual-channel aggregometer (Coulter, Margency, France). The STC of AA, defined as the highest concentration of AA that induced less than 7 % increase in light transmission, varied from one experiment to another and was determined in each experiment. Platelet suspensions were preincubated for 2 min at 37 °C, then incubated with an STC of AA for 30 s at 37 °C in the presence or absence of the HPETE or the derived HETE for another 4 min with continuous stirring at 1000 rev./min. Both AA and HPETEs were added in ethanol and the final concentration of ethanol added to platelet suspensions never exceeded 0.5 %. The extent of platelet aggregation was expressed in terms of percentage of change in light transmission 4 min after the addition of the agonist.

Metabolism of exogenous AA

To determine the incorporation of exogenous AA into lipid classes and its subsequent metabolism, platelets were incubated with an STC of labelled [^{14}C]AA in the presence or absence of HPETE or HETE for 4 min at 37 °C. Platelet lipids were extracted twice with chloroform/ethanol (2:1, v/v) containing 50 μM butylated hydroxytoluene as an antioxidant. Lipid classes were separated by TLC with the solvent mixture hexane/diethyl ether/acetic acid (60:40:1, by vol.) into phospholipids, mono-hydroxylated fatty acids (HHT and 12-HETE), non-esterified fatty acids and neutral lipids (R_f values of 0, 0.20, 0.26, 0.50 and 0.85 respectively) [15]. A second chromatography step was performed with diethyl ether/methanol/acetic acid (90:1:2, by vol.) to separate thromboxane B_2 (TXB_2) (R_f 0.25) from prostaglandins and phospholipids (R_f 0). Distribution into lipid classes and AA metabolites was quantified by radiochromatography with a Berthold TLC linear analyser.

Statistics

Results are expressed as the means $\pm$ S.E.M. or S.D. The statistical significance of differences between different groups was determined by one-way analysis of variance (ANOVA). Statistical difference between two groups was sought by the Fisher protected least-squares difference test.

RESULTS

Effect of AA hydroperoxides on platelet aggregation

As shown in a representative experiment (Figure 1), the addition of 12-HPETE to platelets preincubated with an STC of AA resulted in irreversible aggregation, although 12-HPETE alone had no effect on the platelet response. In contrast, the addition of the hydroxylated derivative 12-HETE to platelets preincubated with an STC of AA did not induce any platelet aggregation.

Increasing concentrations of 12-HPETE ranging from 0.5 to 2 μM potentiated the platelet response to STC of AA in a dose-dependent manner; 1 μM 12-HPETE was the minimum concentration required to enhance platelet aggregation significantly (Figure 2). In contrast, concentrations of HPETEs greater than 2 μM were not able to potentiate platelet aggregation. A positional isomer of 12-HPETE, 15-HPETE, also primed the platelet response to STC of AA with statistical significance reached at 1.5 μM , compared with 1 μM for 12-HPETE (Figure 2).

To determine whether the effect of HPETE was dependent on a metal-mediated formation of radical species from lipid hydroperoxides, the effect of the free-radical scavenger vitamin E and the iron chelator desferrioxamine on the platelet response to 12-HPETE was tested. As reported in Table 1, preincubation of platelets with either 10 μM vitamin E or 2 mM desferrioxamine for 2 min at 37 °C fully prevented the 12-HPETE-induced platelet aggregation.

Figure 1 Effects of 12-HPETE and 12-HETE on the platelet response to AA

Human platelets were preincubated for 2 min at 37 °C and incubated in the presence or absence of an STC of AA for 30 s at 37 °C, followed by the addition of 12-HPETE or 12-HETE for a further 4 min. The percentage aggregation is plotted against time. The results presented are representative of at least six separate experiments.

Figure 2 Effects of different concentrations of HPETEs on the aggregation of platelets co-incubated with an STC of AA

Human platelets were preincubated for 2 min at 37 °C and incubated with an STC of AA ($1.00 \pm 0.07 \mu\text{M}$) for 30 s at 37 °C, followed by the addition of the indicated concentrations of HPETEs [either 12-HPETE (○) or 15-HPETE (◇)]. The extent of platelet aggregation was determined in isolated platelets 4 min after the addition of the agonist. Results are means $\pm$ S.E.M. for six independent experiments. Significance of differences from the respective controls: ** $P < 0.01$; *** $P < 0.001$.

Table 1 Effect of vitamin E and desferrioxamine on 12-HPETE-induced platelet aggregation

Platelets were preincubated for 2 min at 37 °C in the presence or absence of 10 μM vitamin E (Vit. E) or 2 mM desferrioxamine (DFO) followed by the addition of AA (STC = $1.7 \pm 0.2 \mu\text{M}$) and 12-HPETE ($1.3 \pm 0.2 \mu\text{M}$). The extent of platelet aggregation was determined in isolated platelets 4 min after the addition of the STC of AA. Results are means $\pm$ S.D. for three independent experiments. * $P < 0.05$ compared with control platelets (AA).

Addition	Aggregation (%)
AA	2.4 ± 1.8
AA + 12-HPETE	$65.0 \pm 17.0^*$
Vit. E + AA + 12-HPETE	4.2 ± 1.1
DFO + AA + 12-HPETE	2.8 ± 1.8

Table 2 Effect of different peroxides on platelet aggregation

Platelets were preincubated for 2 min at 37 °C, then incubated with an STC of AA (STC = $1.6 \pm 0.4 \mu\text{M}$) for 30 s at 37 °C, followed by the addition of 12-HPETE, tBH or H_2O_2 for 4 min at 37 °C. The results are expressed as the concentration of hydroperoxide causing 50% aggregation (EC_{50}). Each value represents the mean $\pm$ S.E.M. for four independent experiments. * $P < 0.05$ compared with (platelets + AA + 12-HPETE).

Addition	EC_{50} (μM)
AA + 12-HPETE	1.5 ± 0.1
AA + tBH	$4.2 \pm 0.9^*$
AA + H_2O_2	$9.5 \pm 3.1^*$

Table 3 Effects of HPETE (or HETE) on platelet aggregation and exogenous AA metabolism

Platelets were incubated with an STC of [^{14}C]AA ($1.2 \pm 0.1 \mu\text{M}$) in the presence or absence of the indicated HPETE or HETE ($1.5 \pm 0.1 \mu\text{M}$) for 4 min at 37 °C. (a) Maximum aggregation was determined in isolated platelets after 4 min. (b) Platelet lipids were extracted, separated by TLC and metabolites of [^{14}C]AA were quantified by radiochromatography as described. Results are means $\pm$ S.E.M. for at least six independent experiments. ** $P < 0.01$; *** $P < 0.001$ compared with control platelets ([^{14}C]AA).

(a) Platelet aggregation			
Addition	Aggregation (%)		
[¹⁴ C]AA	3.4 ± 1.1		
[¹⁴ C]AA + 12-HPETE	43.1 ± 5.3***		
[¹⁴ C]AA + 15-HPETE	30.6 ± 5.9***		
[¹⁴ C]AA + 12-HETE	0.5 ± 0.5		
[¹⁴ C]AA + 15-HETE	0.3 ± 0.3		
(b) Exogenous AA metabolism			
Addition	[¹⁴ C]AA metabolites (pmol/10 ⁹ platelets)		
	[¹⁴ C]TXB ₂	[¹⁴ C]HHT	[¹⁴ C]12-HETE
[¹⁴ C]AA	92 ± 18	122 ± 18	169 ± 43
[¹⁴ C]AA + 12-HPETE	439 ± 84***	557 ± 99***	442 ± 66***
[¹⁴ C]AA + 15-HPETE	355 ± 80**	416 ± 79**	272 ± 59
[¹⁴ C]AA + 12-HETE	94 ± 13	124 ± 20	181 ± 55
[¹⁴ C]AA + 15-HETE	84 ± 19	97 ± 21	83 ± 15

Effect of non-eicosanoid peroxides on platelet aggregation

To assess the specificity of the platelet response to the AA hydroperoxides, tBH, a model organic hydroperoxide, as well as H_2O_2 , were compared with 12-HPETE: the concentration of lipid hydroperoxides causing 50% aggregation (EC_{50}) of platelets preincubated with STC of AA was determined. As shown in Table 2, the EC_{50} for tBH was 3-fold that for 12-HPETE. Under the same experimental conditions, H_2O_2 was much less potent in priming the platelet response to STC of AA.

Subsequent experiments were performed with HPETEs because they are the most physiologically relevant and potent hydroperoxides under our conditions.

Effects of HPETEs on exogenous AA metabolism

To investigate the effects of HPETEs on the incorporation of AA into phospholipids and its oxygenated metabolism, platelets were preincubated with an STC of [^{14}C]AA followed by the addition of HPETE or HETE. The concentration of HPETE leading to the optimum response, i.e. $1.5 \mu\text{M}$, was selected. The potentiating effect of the HPETEs on platelet aggregation was confirmed (Table 3a). The addition of either 12-HPETE or 15-HPETE induced platelet aggregation significantly, 12-HPETE being slightly more potent than 15-HPETE. 12-HETE and 15-HETE had no effect on the platelet response; the aggregation even tended to be inhibited compared with control platelets. The incorporation of [^{14}C]AA into phospholipids was unaffected by any treatment (results not shown). However, non-esterified [^{14}C]AA tended to decrease in platelets incubated with an STC of [^{14}C]AA and AA hydroperoxides ($2.6 \pm 0.5 \text{ nmol}/10^9$ platelets in control platelets compared with 1.7 ± 0.4 and $1.8 \pm 0.4 \text{ nmol}/10^9$ in platelets co-incubated with 12-HPETE and 15-HPETE respectively), whereas the conversion of [^{14}C]AA into different

Table 4 Effect of aspirin on 12-HPETE-induced platelet aggregation

Platelets were preincubated in the presence or absence of 200 μ M aspirin for 2 min at 37 °C followed by the addition of AA (STC = 1.2 ± 0.0 μ M) and/or 12-HPETE (1.7 ± 0.3 μ M) for 30 s at 37 °C. Platelet aggregation was determined in isolated platelets after 4 min and quantification of [14 C]AA metabolites was performed as described in Table 3. Results are means $\pm$ S.D. for three independent experiments. * $P < 0.05$; *** $P < 0.001$ compared with appropriate controls {aspirin + [14 C]AA compared with [14 C]AA; [14 C]AA + 12-HPETE compared with [14 C]AA; aspirin + [14 C]AA + 12-HPETE compared with [14 C]AA + 12-HPETE}.

Addition	Aggregation (%)	[14 C]AA metabolites (pmol/ 10^9 platelets)		
		[14 C]TXB ₂	[14 C]HHT	[14 C]12-HETE
[14 C]AA	0	115 $\pm$ 54	134 $\pm$ 25	250 $\pm$ 168
Aspirin + [14 C]AA	0	28 $\pm$ 27***	15 $\pm$ 14***	250 $\pm$ 57
[14 C]AA + 12-HPETE	33 $\pm$ 1***	502 $\pm$ 33***	522 $\pm$ 93***	557 $\pm$ 222*
Aspirin + [14 C]AA + 12-HPETE	0***	67 $\pm$ 52***	40 $\pm$ 22***	645 $\pm$ 241

Table 5 Effect of SQ29,548 and Arg-Gly-Asp-Ser (RGDS) on 12-HPETE-induced platelet aggregation and exogenous AA metabolism

Platelets were preincubated in the presence or absence of 200 μ M RGDS for 10 min at room temperature or 100 nM SQ29,548 for 2 min at 37 °C. Platelets were then incubated with [14 C]AA (STC = 1.8 ± 0.4 μ M) and/or 12-HPETE (1.8 ± 0.3 μ M) for 4 min at 37 °C. Results are means $\pm$ S.D. for at least three independent experiments. Maximum aggregation was determined in isolated platelets after 4 min at 37 °C, and quantification of [14 C]AA metabolites was performed as described in Table 3. ** $P < 0.01$; *** $P < 0.001$ compared with appropriate controls {(RGDS or SQ29,548) + [14 C]AA compared with [14 C]AA; [14 C]AA + 12-HPETE compared with [14 C]AA; (RGDS or SQ29,548) + [14 C]AA + 12-HPETE compared with (RGDS or SQ29,548) + [14 C]AA}.

Addition	Aggregation (%)	[14 C]AA metabolites (pmol/ 10^9 platelets)		
		[14 C]TXB ₂	[14 C]HHT	[14 C]12-HETE
[14 C]AA	1.6 $\pm$ 1.2	183 $\pm$ 32	247 $\pm$ 38	242 $\pm$ 11
[14 C]AA + 12-HPETE	48.9 $\pm$ 19.5***	744 $\pm$ 190**	1045 $\pm$ 131***	944 $\pm$ 110***
SQ29,548 + [14 C]AA	0 $\pm$ 0	140 $\pm$ 50	256 $\pm$ 57	206 $\pm$ 22
SQ29,548 + [14 C]AA + 12-HPETE	1.2 $\pm$ 0.6	685 $\pm$ 287***	966 $\pm$ 119***	862 $\pm$ 106**
RGDS + [14 C]AA	1.4 $\pm$ 2.4	153 $\pm$ 24	220 $\pm$ 33	228 $\pm$ 33
RGDS + [14 C]AA + 12-HPETE	3.5 $\pm$ 1.0	705 $\pm$ 221***	1054 $\pm$ 272***	839 $\pm$ 89***

oxygenated metabolites significantly increased in response to the addition of HPETEs (Table 3b). Both [14 C]TXB₂, the stable catabolite of [14 C]TXA₂, and [14 C]HHT, a monohydroxy fatty acid derived from AA via the cyclo-oxygenase pathway, increased by 4.7-fold after the addition of 12-HPETE to platelets preincubated with an STC of [14 C]AA. The cyclo-oxygenase pathway was stimulated more in response to 12-HPETE than to 15-HPETE (4.7-fold increase compared with 3.6-fold increase respectively) but these differences did not reach statistical significance. 12-HPETE also stimulated the 12-lipoxygenase pathway, as assessed by the 2.6-fold increased formation of [14 C]12-HETE. In contrast, 15-HPETE did not significantly alter [14 C]12-HETE formation, presumably owing to its inhibitory effect on the 12-lipoxygenase activity [16]. The addition of the derived hydroxy fatty acids, either 12-HETE or 15-HETE, to platelets preincubated with an STC of [14 C]AA, affected neither the incorporation of AA into phospholipids nor the formation of oxygenated metabolites derived from AA.

Effect of aspirin on 12-HPETE-induced platelet aggregation and exogenous AA metabolism

To determine whether the mechanism of action was dependent on the cyclo-oxygenase pathway, platelet suspensions were preincubated with 200 μ M aspirin before the addition of 12-HPETE and an STC of [14 C]AA (Table 4). The inhibition of the cyclo-oxygenase activity by aspirin was confirmed by the expected inhibited formation of cyclo-oxygenase products, TXB₂ and HHT, and the absence of aggregation induced by aggregatory concentrations of AA. Interestingly, the potentiating effect (by

33 %) of 12-HPETE on platelet aggregation was fully prevented in the presence of aspirin. This inhibitory effect on platelet aggregation was associated with the absence of 12-HPETE-induced increase of TXB₂ and HHT formation, whereas 12-HETE formation was unaffected.

Effect of SQ29,548 and RGDS on 12-HPETE-induced platelet aggregation and exogenous AA metabolism

To confirm that the probable enhancement of the cyclo-oxygenase activity by 12-HPETE and subsequently the increased formation of TXA₂ resulted in enhanced platelet aggregation but not vice versa, a TXA₂ receptor antagonist, SQ29,548, was used. The addition of 100 nM SQ29,548 to platelets co-incubated with an STC of [14 C]AA and 12-HPETE fully inhibited platelet aggregation; the priming effect of 12-HPETE on AA metabolism was sustained (Table 5). Similar results were obtained with 1 μ M SQ29,548.

In addition, the effect of 12-HPETE on the AA cascade in platelets in which aggregation was 'physically' prevented was investigated. As shown in Table 5, the 12-HPETE-induced enhancement of HHT, TXB₂ and 12-HETE formation persisted in platelets preincubated with an STC of [14 C]AA and the tetrapeptide Arg-Gly-Asp-Ser (200 μ M), a potent inhibitor of fibrinogen binding to the glycoprotein IIb/IIIa complex and of aggregation. Similarly, platelets incubated together with AA (STC) and 12-HPETE without any stirring formed comparable enhanced amounts of AA metabolites to the corresponding stirred platelets (results not shown).

DISCUSSION

There is increasing evidence that lipid hydroperoxides, generated by enzymic and non-enzymic pathways, might modulate cell functions and contribute to free-radical-mediated cellular damage in pathological processes. As some pathophysiological states are accompanied by a hyperactivation of blood platelets, it is of interest to determine whether physiologically relevant concentrations of lipid hydroperoxides potentiate platelet function.

The results reported in the present study show evidence of a stimulation of platelet function by hydroperoxides. 12-HPETE, the 12-lipoxygenase-derived hydroperoxide of AA, significantly induced the aggregation of platelets co-incubated with an STC of AA. 15-HPETE, the 15-lipoxygenase metabolite of AA in endothelial cells and leucocytes, also stimulated platelet aggregation but tended to be less efficient than 12-HPETE. Non-eicosanoid peroxides, namely tBH and H₂O₂, were far less potent than AA hydroperoxides. The triggering effect of the hydroperoxy fatty acids on platelet function cannot be due to the derived hydroxy fatty acids, via a possible reduction of HPETE to HETE by intracellular glutathione-peroxidase, because HETEs did not affect platelet function. The fact that vitamin E, an effective scavenger of alkoxyl and peroxy radicals, and desferrioxamine, a powerful chelator of Fe³⁺ ions, prevented the priming of platelet aggregation by 12-HPETE suggests the involvement of radical species derived from the decomposition of HPETEs by traces of transition metals [17] in the effects observed. Under similar experimental conditions, Pratico et al. [18] have shown that H₂O₂ at micromolar concentrations triggered the aggregation of platelets co-incubated with an STC of collagen or AA in a dose-dependent manner and that this effect was mediated by the formation of hydroxyl radicals in an extracellular Fenton-like reaction [19]. However, previous studies have mainly described an inhibitory effect of HPETEs on platelet aggregation. For example, 15-HPETE has been reported to inhibit AA-induced platelet aggregation (IC₅₀ 4–10 µM depending on AA concentration) [10] and 12-HPETE has been shown to inhibit platelet aggregation in a concentration-dependent fashion, the IC₅₀ values ranging from 3 to 6 µM depending on the agonist used [11]. Whereas the inhibition of platelet aggregation occurred at relatively high concentrations, our results provide evidence that HPETEs can prime platelet aggregation when used at doses closer to physiological concentrations (1–2 µM); such concentrations can be reached in platelets stimulated with 0.1 unit/ml thrombin [5]. This contention is supported by previous studies showing that mildly oxidized low-density lipoproteins (LDL) caused a direct aggregation of platelets or oxidized LDL increased the sensitivity of platelets to agonists [20]. Interestingly, these effects seemed to be dependent on the extent of lipid peroxide formation. Mildly oxidized LDL containing low concentrations of lipid peroxides were indeed more potent than strongly oxidized LDL with high lipid peroxide concentrations in terms of platelet activation. Another difference is that platelets needed to be co-incubated with an STC of agonists to obtain an amplification of the platelet response. Such conditions are likely to be encountered *in vivo* as circulating platelets might be exposed to increasing concentrations of agonists near the site of an injured vessel wall. Interestingly, 12-HPETE (or 15-HPETE) was also able to potentiate the aggregation of platelets co-incubated with an STC of an agonist such as collagen, which reinforces the physiological relevance of the data (results not shown).

Several mechanisms can account for the enhancement of platelet function by the HPETEs; those relating to the AA cascade were considered. The priming effect of 12-HPETE on AA metabolism was not prevented by SQ29,548 or RGDS,

indicating that the HPETE-induced enhancement of platelet aggregation is not mediated by a thromboxane or a glycoprotein IIb/IIIa receptor. The potentiating effect of 12-HPETE on platelet function was closely associated with an increased formation of cyclo-oxygenase metabolites, and in particular TXA₂ production. Aspirin both prevented the increased platelet aggregation in response to an STC of AA and the increased formation of cyclo-oxygenase-derived compounds, confirming that the HPETE-induced enhancement of platelet function is mediated by a stimulation of the cyclo-oxygenase activity. It could constitute the most likely mechanism of activation by HPETEs of platelets preincubated with an STC of AA because the addition of exogenous AA bypasses the activation of phospholipases to induce TXA₂ generation. It is then probable that the activation of the AA cascade initiated by an STC of AA might have been amplified by triggering concentrations of HPETEs, via a stimulation of the cyclo-oxygenase activity. These results corroborate the requirement of a hydroperoxide initiator for the activation of cyclo-oxygenase [21]. Moreover it has recently been shown that the cyclo-oxygenase activity of the sheep constitutive isoform PGHS-1 was activated by nanomolar levels of hydroperoxides [22]. Although doses of HPETEs necessary to potentiate platelet functions were higher in our conditions than those required to activate the cyclo-oxygenase activity in purified preparations of PGHS-1, one cannot rule out a lower concentration of HPETE actually available in our cellular model owing to the presence of the efficient glutathione-peroxidase in intact platelets. In contrast, the cyclo-oxygenase activity might also be inhibited by micromolar concentrations of lipid hydroperoxides, which is consistent with the reported inhibitory effects of such concentrations of HPETEs on platelet function [10,11,23]. As regards the 12-lipoxygenase pathway, the addition of 12-HPETE to platelets incubated with an STC of AA also increased 12-HETE formation derived from exogenous AA, which agrees with previous data reporting a stimulation of platelet 12-lipoxygenase activity by 12-HPETE [9,24]. In contrast, 15-HPETE did not increase 12-HETE formation because it is a potent inhibitor of platelet 12-lipoxygenase [16]. The opposite effects of the two hydroperoxides tested on the 12-lipoxygenase activity might explain in part the lower potency of 15-HPETE compared with 12-HPETE because endogenous 12-HPETE might have contributed to the stimulation of cyclo-oxygenase activity together with exogenous 12-HPETE.

In conclusion, the results of the present study indicate that physiologically relevant concentrations of AA hydroperoxides prime the aggregation of platelets co-incubated with non-aggregating concentrations of AA and that this effect could be mediated via a stimulation of the cyclo-oxygenase activity. It thus seems that HPETEs might contribute to platelet hyperfunction observed in some pathophysiological states such as thrombosis and atherosclerosis.

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