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DIFFERENTIAL EFFECTS OF 15-HPETE ON ARACHIDONIC ACID METABOLISM IN COLLAGEN-STIMULATED HUMAN PLATELETS

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The 15-hydroperoxyeicosatetraenoic acid (15-HPETE) has been shown to affect platelet aggregation induced by collagen, arachidonic acid (AA), and PGH_2-analogue. Furthermore, it also inhibits the platelet cyclooxygenase and lipoxygenase enzymes, and prostacyclin synthase. The present study was designed to test the effect of 15-HPETE on the mobilization of endogenous AA in collagen-stimulated human platelets. For this purpose, human platelets pretreated with BW755C (a dual inhibitor of cyclooxygenase and lipoxygenase) were stimulated with collagen in the presence of varied concentrations of 15-HPETE. We observed a significant inhibition of oxygenases at all concentrations of 15-HPETE. In contrast, our results indicate that 15-HPETE at lower concentrations (10 μM and 30 μM) significantly stimulated the collagen-induced release of AA from phospholipid sources. Although higher concentrations of 15-HPETE (50 μM and 100 μM) caused some inhibition of AA accumulation in the free fatty acid fraction (25% and 60%), the degree of inhibition was significantly lower than the inhibition observed for the oxygenases (65% and 88% for cyclooxygenase and 77% and 94% for lipoxygenase respectively). These results provide support that hydroperoxides also regulate phospholipases presumably by a different mechanism, which may be important in the detoxification of phospholipid peroxides.

Mammalian cells metabolize AA via the cyclooxygenase and lipoxygenase enzymes to hydroperoxides, which are further reduced to the corresponding stable metabolites by peroxidases (1). There is increasing evidence to suggest that the fatty acid hydroperoxides (FAHP) in living tissues can have implications in the pathophysiology of several diseases. Furthermore, it has been shown that small amounts of FAHP are required for activation of the cyclooxygenase complex (2). However, these hydroperoxides cause irreversible inactivation of the enzyme at higher concentrations (1). In other words, the hydroperoxides derived from AA actively participate in the regulation of

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cyclooxygenase/lipoxygenase activities.

The rate of production of eicosanoids by cyclooxygenase/lipoxygenase is also limited by the availability of precursor AA from membrane phospholipids (3-6). When platelets are exposed to external stimuli, lipases such as diacylglycerol lipase and phospholipase A_2 undergo rapid activation and cleave off AA from membrane phospholipids (7,8). In addition, it has recently been suggested that phospholipase A_2 may play a crucial role in the detoxification of phospholipid peroxides by selectively removing them from the oxidized membrane (9).

Human platelets upon activation with collagen release AA from membrane phospholipids, and the released fatty acid is then metabolized to prostaglandins, thromboxanes, and 12-hydroxyeicosatetraenoic acid (12-HETE) (10). It appears that endoperoxides (PGH_2) and thromboxane A_2 play a pivotal role in collagen-mediated platelet activation (11-18). Furthermore, it has been suggested that the lipoxygenase metabolite, 12-HETE, may regulate platelet aggregation and secretion, and may possibly interfere with the endoperoxide-induced responses (19). The present study was designed to test the hypothesis that FAHP may also be involved in the regulation of phospholipases in stimulated intact platelets.

MATERIALS AND METHODS

Materials: The tritium labelled AA and aquasol were purchased from the New England Nuclear Corp. (Boston, MA). Collagen preparations were obtained from Hormone Chemie (FRG). 15-HPETE was donated by C.C. Reddy (State College, PA). 3-Amino-1-(3-Trifluoromethylphenyl-2-Pyrazoline Hydrochloride (BW755C), a dual inhibitor of cyclooxygenase/lipoxygenase was donated by the Wellcome Research Laboratories (Kent, UK). AA metabolites and lipid standards were obtained from the Sigma Chemical Co. (St. Louis, MO). Pre-coated thin layer silica gel plates (H60) were purchased from E. Merck (Darmstadt, FRG). All solvents and chemicals employed were of analytical grade.

Isolation, Prelabelling and Preparation of Platelet Suspensions

Blood samples from the antecubital veins of human volunteers who had abstained from taking any anti-inflammatory drugs including aspirin were drawn into siliconized vacutainer tubes containing 1/10 volume of acid citrate dextrose (ACD). Platelet-rich plasma (PRP) obtained by centrifugation at 200 x g for 7 min was incubated with labelled arachidonic acid (2.5 μCi/15 ml of PRP) at 37°C in a shaking water bath for 2 hr. Labelled platelets were then isolated from the PRP according to the method of Mustard et al. (20), and the platelets were finally suspended in Tyrode-albumin solution containing calcium (2 mM). The platelet counting was

performed in a Coulter Counter, and the cell density was adjusted to $5x10^8$ platelets/ml.

Incubation of Platelet Suspensions

In the first set of experiments, the labelled platelet suspensions were stimulated with collagen (100 µg/ml) in the presence of varied concentrations of BW755C. This study allowed the determination of the effects of BW755C on cyclooxygenase/lipoxygenase activities in collagen-stimulated intact cells. In the second set of experiments, the platelet suspensions were incubated with varied concentrations of 15-HPETE and then exposed to collagen in order to assess the effect of 15-HPETE on the collagen-induced formation of thromboxane B_2 (TxB_2) and 12-HETE. In the third set of experiments, the platelet suspensions were preincubated for 1 min at 37°C in a metabolic shaker, and BW755C (dissolved in water) and 15-HPETE (dissolved in ethanol) were added to the platelet suspension at 1 min and 2 min respectively. In these incubations, a known concentration of BW755C (75 µM) which caused about 92-94% inhibition of cyclooxygenase/lipoxygenase activities and exerted no significant inhibitory or stimulatory effect on agonist-activated phospholipases, was employed. Collagen was added at 3 min (100 µg/ml) and the suspensions were left in the presence of collagen for another 3 min. At the end of these incubations, appropriate extraction solvents were added, and metabolites and lipids were extracted and analyzed as described in the following sections.

Extraction and Analysis of AA Metabolites

AA metabolites were extracted from the incubation medium by the addition of 3% formic acid, and subsequent extraction with diethylether. The ether extracts containing AA metabolites were dried under nitrogen and resuspended in chloroform:methanol (2:1, v/v). Thin-layer chromatographic analysis of these metabolites was performed on silica gel H plates using the solvent system described by Salmon and Flower (21). The bands corresponding to TxB_2 and 12-HETE were scraped and the radioactivity determined by a liquid scintillation counter (Beckman LS9800).

Extraction and Analysis of Free AA

Lipids from platelets incubated with BW755C, 15-HPETE and collagen were extracted immediately by the addition of chloroform:methanol (1:2, v/v), and processed further according to the method of Bligh and Dyer (22). The lower chloroform phases were dried under nitrogen and redissolved in chloroform: methanol (2:1, v/v). The lipid extracts were used for the analysis of phospholipids, newly formed phosphatidic acid and AA by two dimensional thin-layer chromatography using the solvent systems described before from our laboratory (23). The bands corresponding to free AA were scraped and counted for radioactivity in a liquid scintillation counter.

RESULTS AND DISCUSSION

Activation of platelets with collagen results in the stimulation of phosphoinositide metabolism, an elevation of cytoplasmic calcium, and protein phosphorylations (11-18). Furthermore, collagen stimulates the mobilization of AA from phosphatidylcholine (PC) via the action of phospholipase A_2 and possibly from diacylglycerol via the sequential action of diacylglycerol and

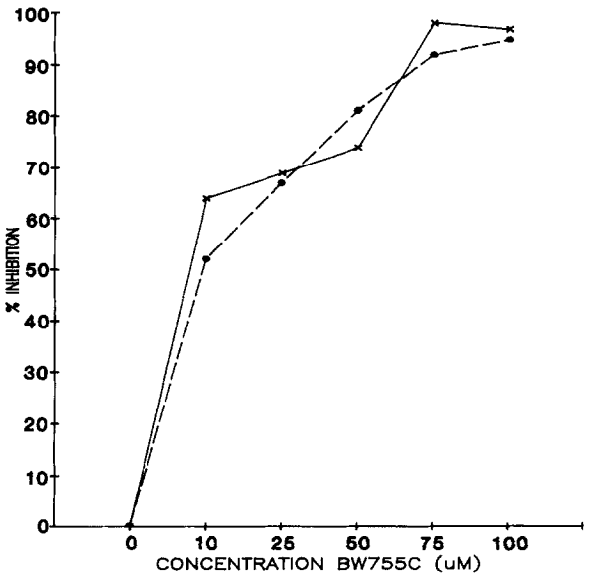

Fig. 1. [^{3}H] AA labelled human platelets ($5x10^8$ platelets/ml) were treated with different concentrations of BW755C for 1 min and then stimulated with collagen (100 µg/ml) for 3 min at 37°C. The arachidonic acid metabolites (TxB_2 and 12-HETE) formed in response to collagen were immediately extracted and analyzed as described in Materials and Methods. x —— x, TxB_2; o ---- o, 12-HETE. Results are expressed as the percent inhibition.

monoacylglycerol lipases (7,8,13). The released AA is then converted to TxB_2 and 12-HETE via the action of cyclooxygenase and 12-lipoxygenase respectively.

In this report, the effect of 15-HPETE on the mobilization of endogenous AA from membrane phospholipids following stimulation with collagen in intact human platelets was studied. In order to examine this question, it was essential that the released AA was not further converted to its metabolites prior to the addition of 15-HPETE. We have consistently found that BW755C inhibits the cyclooxygenase and lipoxygenase enzymes without significantly affecting the mobilization of endogenous AA. Figure 1 shows the effect of BW755C on TxB_2 and 12-HETE formed upon stimulation of platelets with collagen. As shown in this figure, the percent inhibition of cyclooxygenase and lipoxygenase enzymes achieved at different concentrations of BW755C in platelets exposed to collagen is in general agreement with previous reports (24,25).

For comparative purposes, it was also essential to establish the effect of 15-HPETE on the activities of cyclooxygenase and lipoxygenase enzymes. The results obtained on the effect of 15-HPETE are presented in Table 1. 15-HPETE

Table 1. Effects of 15-HPETE on the formation of TxB_2 and 12-HETE in collagen-stimulated human platelets

15-HPETE Concentration	TxB_2	12-HETE
Control	128 ± 12	231 ± 14
0 μM	2965 ± 30 (0%)	5560 ± 132 (0%)
10 μM	1036 ± 6 (68%)*	1928 ± 30 (68%)*
30 μM	1583 ± 1 (49%)*	2168 ± 42 (64%)*
50 μM	1115 ± 10 (65%)*	1462 ± 22 (77%)*
100 μM	465 ± 26 (88%)*	561 ± 26 (94%)*

[^{3}H]AA labelled human platelets ($5x10^8$ platelets/ml) pretreated with different concentrations of 15-HPETE were stimulated with collagen (100 μg/ml) for 3 min at 37°C. No collagen was added to the control. The AA metabolites were extracted and analyzed as described in Materials and Methods. The radioactivity of TxB_2 and 12-HPETE is expressed as absolute dpm values. The numbers in the parenthesis indicate the percentage of inhibition. Results are expressed as the means ± S.D. (n=2). *Values are significantly different from the control ($P<0.05$).

inhibited both the cyclooxygenase and lipoxygenase activities at all concentrations and the degree of inhibition was 68% at the lowest concentration employed in this study (10 μM). Vanderhoek et al. (26) have reported the IC_{50} values as 5.7±0.3 and 2.5±1.0 for 15-HPETE on platelet cyclooxygenase and lipoxygenase enzymes, respectively. In addition, Vericel and Lagarde (27) have shown that 15-HPETE inhibits AA and PGH_2 analogue-induced platelet aggregation. 15-HPETE also inhibits prostacyclin synthase (28,29). The effect of 12-HPETE, the main lipoxygenase metabolite on AA metabolism, has also been studied by several laboratories (19,26,30,31).

Collagen is a unique stimulus in view of the fact that the responses induced by this stimulus are dependent on the metabolites derived through cyclooxygenase (11-13,32). However, Pollock et al. (13) have recently reported that collagen can stimulate a significant release of AA from PC presumably by a Ca^{++}-dependent phospholipase A_2. In addition, the changes that occur in phospholipid molecular species following stimulation of human platelets with collagen have been documented (33). Our own data on collagen-induced phospholipid changes confirm these findings (unpublished

Table 2. Effects of 15-HPETE on [^{3}H]AA accumulation in collagen-stimulated human platelets

15-HPETE Concentration	[^{3}H]AA release
0 μM	13,463 ± 31 (100%)
10 μM	14,487 ± 438 (108%)*
30 μM	15,383 ± 156 (114%)*
50 μM	10,111 ± 291 (75%)*
100 μM	5,333 ± 500 (40%)*

[^{3}H]AA labelled human platelets ($5x10^8$ platelets/ml) were treated with BW755C (75 μM) and then with different concentrations of 15-HPETE (0-100 μM) followed by stimulation with collagen (100 μg/ml) for 3 min at 37°C. Free [^{3}H]AA was extracted and analyzed as described in Materials and Methods. The results are expressed as the means±S.D. (n=2). *At 10 μM and 30 μM, the accumulation of [^{3}H]AA was significantly stimulated ($P<0.05$) whereas at 50 and 100 μM, it was significantly inhibited ($P<0.05$).

observations). Moreover, it has been shown that a significant amount of AA is mobilized in response to collagen from membrane phospholipids (PC) in the presence of an inhibitor of cyclooxygenase (indomethacin) (13) and an inhibitor of cyclooxygenase and lipoxygenase enzymes (BW755C) (Table 2). Thus, the use of BW755C in our incubations prior to the addition of collagen facilitated the accumulation of AA in the free fatty acid fraction irrespective of the phospholipid sources. In other words, the lipid pool that was affected in our experiments by collagen to release free AA is independent of the cyclooxygenase and/or lipoxygenase metabolites.

As shown in Table 2, the results indicate that the effects of 15-HPETE on AA release are different from those observed on the cyclooxygenase/ lipoxygenase activities. We observed a substantial stimulation of AA release at lower concentrations of 15-HPETE (10 μM and 30 μM) and the corresponding stimulation amounted to 108% and 114% of the control (the actual release of AA is treated as 100%). In contrast, both cyclooxygenase and lipoxygenase enzymes were inhibited significantly. The degree of inhibition for the cyclooxygenase was 68% and 49% at 10 μM and 30 μM whereas it was 68% and 64% for the lipoxygenase respectively. This differential effect of 15-HPETE on AA

release was evident even at higher concentrations. The release of AA was inhibited moderately (25%) at 50 μM whereas the cyclooxygenase and lipoxygenase enzymes were inhibited to the extent of 65% and 77% respectively. At 100 μM the inhibition of AA release by 15-HPETE was only 60% whereas the inhibition of cyclooxygenase and lipoxygenase amounted to 88% and 94% respectively. The inhibition of AA release observed at 50 μM and 100 μM may be in part due to the inhibition of diacylglycerol lipase. It has been shown that 15-HPETE inhibits diacylglycerol lipase in broken platelet preparations (34). In conclusion, the effect of 15-HPETE on AA release was different from the effect it exerted on the oxygenases.

This is the first demonstration that FAHP could exert such differential effects on AA metabolism in intact platelets. The dual role of FAHP as observed in this study suggests their importance in the regulation of phospholipases. The work of van Kuijk et al. (9) has provided evidence for preferential removal of the peroxidized fatty acid from phospholipid substrates by phospholipase A_2. Although our experimental approach did not address this question, the differential effect of 15-HPETE on AA release observed in this study suggest an important regulatory role for phospholipases that are responsible for the mobilization of endogenous AA.

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